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THE MOLECULAR ASPECTS OF BIOLOGICAL DEVELOPMENT

Edited by R. A. Deering and Muriel Trask

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THE PENNSYLVANIA STATE UNIVERSITY
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THE MOLECULAR ASPECTS OF BIOLOGICAL DEVELOPMENT

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A Workshop

Held at the Pennsylvania State University, University Park, Pa.

July 19 - 21, 1965

Sponsored by

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Preface

This is the transcript of an informal workshop on "The Molecular Aspects of Development" held at the Nittany Lion Inn of The Pennsylvania State University, University Park, Pennsylvania, on July 19-21, 1965. It was organized by The Pennsylvania State University Biophysics Department under the sponsorship of the University and the National Aeronautics and Space Administration. Its purpose was to bring together scientists actively doing research in different areas of differentiation and development. Researchers from several disciplines doing work on many different biological systems were invited to give presentations of their work. These presentations were informal and discussion was invited at all times. In addition to those invited to give talks, participants were invited from many related departments at the University. A complete list of all participants is given following this preface.

This conference reflects the growing interest in the problems of differentiation and development as attacked from the molecular point of view. The combined backgrounds and methods of many disciplines such as biochemistry, biophysics, cell biology, genetics, microbiology, physical chemistry, physics, mathematics and others are being brought to bear on this problem and the potential reward is great. An interdisciplinary approach to this problem is necessary and should be emphasized. Free informal communication between scientists with differing backgrounds and viewpoints is essential. We feel that this conference was a successful step in that direction and as such was valuable to all participants. We hope that this publication of the presentations and discussions will be useful to the student, teacher and researcher who is interested in the problem of development in biological systems.

The conference was taped and transcribed. Each participant was then given a chance to rework his contribution, with the directive to retain the informality and to leave spontaneous discussion intermixed with presentations. The slides and blackboard drawings used in most presentations have been reproduced here as figures, some of which were redrawn from photographs of the projected slides or sketches furnished by the authors. These are sometimes incomplete and are merely used to illustrate points in the talks. More complete data can often be found in the original publications which are referenced throughout. The attempt to retain the spontaneous, informal flavor of the workshop may result in some presentations seeming incomplete and unpolished. However, since spontaneity and informality are the values of a conference of this type, we feel the reader should be allowed as true a view of it as possible.

We wish to thank those who made this conference and publication possible: in particular, the National Aeronautics and Space Administration (Grant NsG-324), through the efforts of Dr. George J. Jacobs, Chief, Physical Biology Biosciences Program; The Pennsylvania State University Biophysics Department and its chairman, Dr. Ernest C. Pollard; and The Pennsylvania State University College of Science and its Dean, Dr. C. I. Noll. We are indebted to Dr. Paul Ts'o for his extra effort in preparing the summary presentation which appears at the end. Finally, we wish to thank all participants for their enthusiastic discussion and their cooperation and hard work in preparing presentations and manuscripts.

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May 4, 1966

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RNA AND PROTEIN SYNTHESIS IN DEVELOPING SEA URCHIN EGGS

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I propose to summarize here what I believe are some important points emerging from the recent study of biochemical events, especially those involved with macromolecule synthesis, that follow immediately after fertilization of sea urchin eggs (1). There appears to be a necessity for the existence of systems controlling protein synthesis at the level of translation of RNA messages (2). Experiments on the early course of development are now no longer unique in demonstrating the existence of translation control. However, in fact, these were among the first in which the necessity for such a conclusion appeared. There are a number of other kinds of developing and differentiating systems in which evidence of control at this level is available. Some of these will undoubtedly be considered as these discussions progress.

The observations that led to the postulation of translation control follow. Synthesis of proteins, which is an inevitable accompaniment of early development, may be uncoupled from the synthesis of new RNA (e.g., 3). This uncoupling can be absolute and may last for a very long time. When the observation was first made, it was surprising because the situation with respect to messenger function of RNA in microbial cells would not necessarily have led to the prediction of such a level of control, since in microbes the continuation of protein synthesis requires concomitant synthesis of RNA messages whose half life is short, relative to the length of the cell cycle. In a system allowing the synthesis of protein to go on in the absence of new synthesis of messenger RNA, it must be true that either such synthesis doesn't require messages, or that the messages are very stable.

The possibility that protein synthesis accompanying early development may not require messenger RNA could be established in a number of ways. One could, for example, look for polyribosomes in embryos. One could make estimates of the fraction of the early synthesis that occurs on polyribosomes, and if most of the synthesis does occur there, then it is reasonable to assume that protein synthesis does require messenger RNA and associated ribosomes. Such seems to be the case (4, 5, 6). There is no primary site that we have been able to detect for protein synthesis in sea urchin eggs other than ribosomes associated with a length of highly nuclease-sensitive RNA. The extent to which those objects, themselves, are associated with other, perhaps larger, structures is an interesting point that I hope will come up in the discussion later. At least, the polyribosome, itself, is the unit on which early proteins are made. Since under uncoupling conditions new messages are not made, old ones must supply the information for translation. That much alone suggests that these messages must be stable. Although at the time the observations were made, that was in itself a moderately radical proposal, the existence of very stable messages has since been shown in several cases (e.g., 7). Stable messages seem now to be not at all exceptional in higher cells, even in relation to the long inter-mitotic time.

Our starting point was the independence of new protein synthesis from new RNA synthesis in embryos; that is, messages directing the early synthesis must have been present in the egg before it was fertilized. There is, quite generally, a long period between the time that an egg is completed and set aside in a condition of relative dormancy in the ovary, and the time it is released from the mother to be fertilized. Hence, the further suggestion that the templates for early embryonic protein synthesis are not only very stable in use, but may be stored for

long periods of time without being used at all. There are several kinds of developing and differentiating systems to which the statements I have just made are now known to apply. This being so, we conclude that some agency of control must exist in the cytoplasm to turn on the reading of stored messages, since it is demonstrable in all of the systems being studied that the co-factors necessary for protein synthesis are already available in the unfertilized egg. Thus, there has emerged from studies of macromolecule synthesis in development a need to find out how translation-control systems work. They clearly exist and they must be concerned not only with the control of development but with the control of decision-making processes, in general, in differentiated higher cells.

As far as I know, there is no detailed scheme that explains as yet how any translation-control system works. Perhaps we will have suggestions in the course of this week, as to where to look for the agencies of control. In the meantime, there are experiments that led to the position I have just sketched, which lead in turn to a closer study of the events of macromolecule synthesis in early development. I will discuss three lines of such experimentation briefly, relying mainly upon slides to summarize the present position in each case. The three problems with which we will be concerned are (1) the pattern of synthesis of RNA during early development, (2) the search for stored maternal messages whose existence is suggested although not proven by indirect evidence and (3) a study of the proteins themselves, a large fraction of which presumably are made on

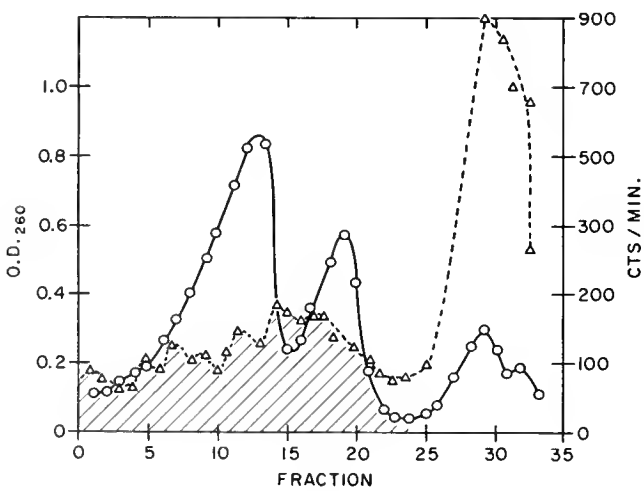

Fig. 1.

stable messages during the period of cleavage.

The pattern of RNA synthesis is radically different from what one might have expected from the behavior of microbial systems. Figure 1 deals with a sucrose gradient and with RNA labeled for 30 minutes at the blastula stage in the sea urchin embryo. I have chosen this pattern to start with because it is characteristic of the pattern of synthesis of RNA throughout the course of the period from cleavage to the late blastula. The sea urchin has ordinary RNA in bulk, with 28S, 18S and 4S species. (These are the three major peaks of Fig. 1 from left to right, respectively, shown by circles.) Radioactivity incorporated in this case from labeled uridine is distributed in gradients as shown by the triangles. The circles are O. D. Such radioactive material is non-coincident with the stable pre-existing bulk RNA, except in the 4S region. The radioactive product is highly heterogeneous with respect to sedimentation constant. In the 4S region, where coincidence does occur, there is also, throughout early cleavage, the most rapidly labeled RNA. There is every reason to suspect, on the basis of physical behavior alone, that the non-4S material being labeled is not ribosomal and is very likely, at least, to be messenger RNA, or heterogeneous RNA with possible template function. I should point out that in these embryos there are no nucleoli until long after the swimming blastula stage. Figure 2 is a fortunate tangential cut through the surface of a quite late blastula, already ciliated and swimming. It shows nuclear profiles. Note that there are no nucleoli. As long as there are none, we see little or no ribosomal RNA synthesis on gradients, no incorporation of label that sediments in coincidence with the ribosomal species and, as we shall see in a moment, base compositions for the newly-synthesized material that differ radically from those of the bulk ribosomal RNA. When the nucleoli do appear at the late gastrula stage, it becomes possible to detect ribosomal RNA synthesis at a steadily increasing rate.

Figure 3 is another experiment like the one represented in Fig. 1, but in this case the labeling was with radioactive phosphate. Four sets of fractions were pooled, corresponding roughly to the centers of gravity of the 28S, 18S, 10S and 3-1/2S bulk RNA. Base-composition analyses were performed.

Table I will show what the compositions are. The fractions shown here were indicated in Fig. 3. This work was done with *Arbacia*. DNA of this species has a GC content of slightly

TABLE I

Base Compositions of Sea Urchin RNA Fractions[a]

Sample	(Mole %) A	U	G	C	(Mole %) G + C	Source
Fraction I	28.9	24.4	23.6	23.1	46.7	These exp'ts.
Fraction II	28.1	27.4	21.8	22.3	44.6	"
Fraction III	33.8	16.2	18.2	31.8	50.0	"
Fraction IV	14.4	12.9	14.5	58.2	72.7	"
28S rRNA	22.4	18.8	32.8	26.0	58.8	Gross, Malkin & Hubbard (1965)
18S rRNA	24.4	21.7	30.0	24.0	54.1	"
Bulk RNA	22.3	20.7	29.6	27.4	57.0	Elson, et al. (1954)
Arbacia DNA	28.4	32.8(T)	19.5	19.3	38.8	Daly, et al. (1950)

Fractions I-IV from *Arbacia* embryos exposed to $^{32}PO_4$ from fertilization to early blastula. 28S and 18S RNA from *Arabacia* eggs labeled with $^{32}PO_4$ during oogenesis. Hydrolysis, separation of nucleotides and determination of base composition according to Salzman, Shatkin and Sebring (1964), except for compositions of bulk (total) RNA and DNA (sperm), which are from the literature. Approximate centers of gravity in sedimentation profile corresponding to fractions I-IV: 28S, 18S, 10S, 3S.

[a] Table I, Gross, Kraemer and Malkin, *Biochem. Biophys. Res. Comm. 18*, 569, 1965; reproduced with permission of Academic Press.

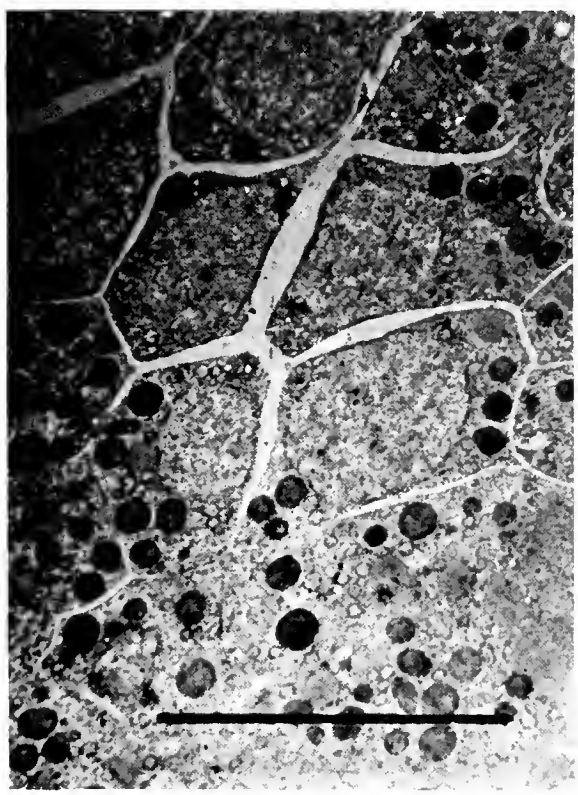

Fig. 2.

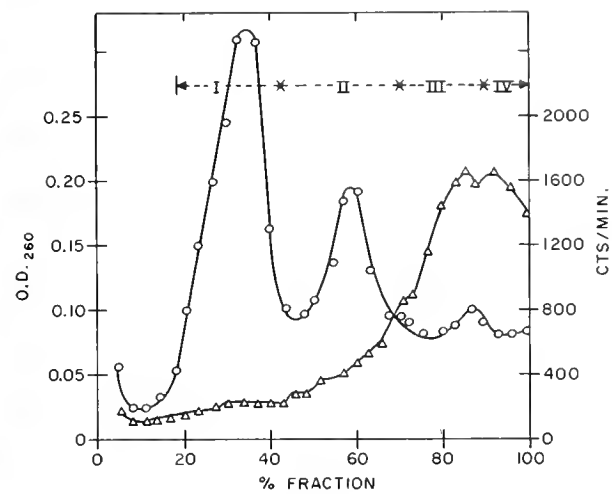

Fig. 3.

(Fig. 1, Gross, Kraemer and Malkin, *Biochem. Biophys. Res. Comm. 18*, 569, 1964; reproduced with permission of Academic Press.)

under 40%. The bulk RNA, mainly the two ribosomal species, has a GC content of about 57%. RNA labeled through early cleavage up to the blastula and all fractions except the lightest one have the GC content that is markedly lower than what would be expected for ribosomal RNA. Remember that this is accumulation of radioactivity over a period of about seven hours with the radio-phosphate in the medium being kept at constant specific activity, so that with respect to GC content, this is very much a DNA-like RNA and probably one of considerable stability.

The base composition of the light fraction is highly aberrant. It is very rich in cytidylic acid, and has a roughly equal distribution of radio-activity among the other bases. This suggests strongly that the heavy incorporation of radioactivity coincident with the 4S peak represents labeling of the terminal CCA sequence in transfer RNA. This is the dominant synthetic process associated with RNA in the course of earliest development and far outweighs the activity associated with internal synthesis. What the significance of the end-labeling is, I do not know, and I have not heard any really useful suggestions about it. It seems to be a widespread phenomenon in developing systems and in other systems in which cells do not grow. I should point out in this connection that the embryos don't grow in any strict sense. New cells are forming as a result of cleavage, but there is no increase in mass. Indeed, throughout the course of development to the early larval stages, there is a slow but

Table II (top) shows the base composition for fractions α, β, γ, δ, ε as indicated in Fig. 4. Remember that the DNA has a 40% GC content and that these are pooled fractions from heavy to light. Most of them still have a low GC content except that as one approaches the light end, GC content rises because there is still a considerable amount of end-labeling. There is clearly some ribosomal RNA accumulating during this period. If incorporation is allowed to take place from the late gastrula to the prism stage, as shown by Fig. 5 (symbols as for Fig. 4), which is the beginning of the differentiation of definitive larval tissues, then there is a predominant ribosomal RNA synthesis, quite steady decline in mass, and this is because some carbon compounds are broken down to CO_2 and water.

If one allows radioactivity to be incorporated into RNA later, for example, with P^{32} as the label (Fig. 4), from late blastula to early gastrula, using a long labeling period (about seven hours), one gets something that looks as though there were the beginning of ribosomal synthesis. (Open circles, OD; closed circles, counts per minute; triangles, specific activity.) Notice that the specific activities are minima where there are optical density maxima, suggesting that coincidence is poor between the bulk ribosomal RNA (represented by optical density) and the radioactivity. The base composition again shows that the heterogeneous RNA is still present even after a long exposure to isotope.

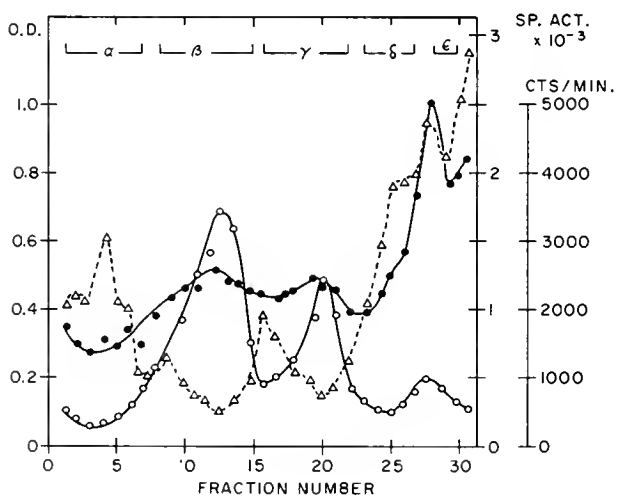

Fig. 4.

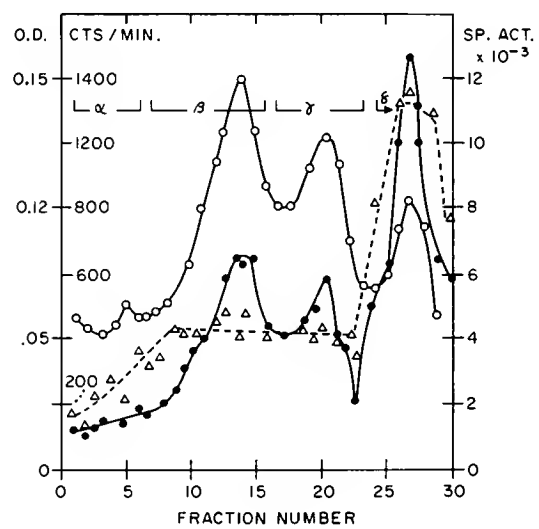

Fig. 5.

TABLE II

Base Composition of RNA Synthesized During Long Exposures of Sea Urchin Embryos to ^{32}P.

Sample	Fraction	Composition, Mole % A	U	G	C	% G + C
Blastula	α	29.7	23.1	25.6	21.5	47.3
Gastrula	β	27.7	25.7	29.1	17.5	46.6
7 hrs.	γ	32.6	23.8	23.0	20.6	43.6
	δ	23.9	23.5	26.2	26.3	52.5
	ε	18.8	18.0	32.3	30.9	63.2
Gastrula	α	25.7	26.1	23.6	24.6	48.6
Prism	β	23.9	23.2	27.2	25.7	52.9
12 hrs.	γ	25.4	22.5	26.5	25.6	52.1
	δ	21.5	22.1	28.4	27.9	56.3
*18S rRNA		24.4	21.7	30.0	24.0	54.1
*28S rRNA		22.4	18.8	32.8	26.0	58.8

represented both by the change in base composition (Table II, lower part) and by a clear coincidence of the counts with the absorbancy pattern. (Notice the constant specific activity across the ribosomal optical density peak in Fig. 5.) Thus RNA synthesis begins in this system under conditions such that little or no ribosomal RNA is made and the major incorporation activity represents labeling of the CCA terminal in transfer RNA. In time, the rate of end-labeling falls and the rate of synthesis of heavy heterogeneous RNA rises steadily from fertilization onward. At some point, probably well after the blastula and perhaps as late as the time of appearance of definitive nucleoli, the synthesis of ribosomal RNA begins in quantity. This means that a complicated system of control operates on the synthesis of RNA, and specifically, on the utilization of the cistrons that provide templates for synthesis of the ribosomal RNA.

This is all heavily descriptive, and I cannot offer anything in the way of a reasonable explanation for the existence of this pattern, but it is beginning to be quite a general one. For example, the situation in the amphibian seems to be roughly the same, except that there is some argument about when the synthesis of new heterogeneous RNA begins. Dr. Kohne will tell you about this later.

POLLARD: I would like to ask you a couple of questions. First, as a microbiologist, I'd like to know what the amount of turnover of RNA and protein is. In *E. coli*, for example, the RNA does turn over to some extent. At least the uracil label changes.

GROSS: Does it turn over in the ribosomal RNA?

POLLARD: I don't think it does, but if we just look at the general cell behavior, there is a difference between thymine label and uracil label. To what extent do you see something like that?

GROSS: There are two different answers, depending on how you evaluate the available data. Comb (8) believes that there is some considerable degradation of ribosomal RNA in sea urchin embryos from the beginning of development to the gastrula stage, and that the products of degradation are possibly used for resynthesis of messenger RNA. That is the only information I know that suggests such a turnover. Other data that we have do not offer much support for this idea. The egg starts its life with a large pool of precursors for RNA, and this pool diminishes slowly but steadily

during development. It doesn't enlarge, as far as I can tell, at any time. Actual synthesis of bulk RNA, represented at least by the incorporation of radioactivity, seems to be small, perhaps not exceeding a few per cent of the original total. Certainly on this basis there is no need for massive synthesis of bulk RNA. Finally, by a technique that I'll describe in a minute, we have been able to label the RNA in ribosomes of unfertilized eggs. If such eggs are fertilized and allowed to develop in the presence of a very large excess of unlabeled uridine and cytidine in the medium, there is no detectable loss of counts in the RNA. This is probably in inadequate "chase" and certainly not direct evidence for the complete stability of cytoplasmic RNA. There is, in short, no really adequate answer to your question at this moment, but one's prejudice is in the direction of little or no turnover.

POLLARD: How about the case of protein synthesis?

GROSS: That may really be the more interesting matter. The egg starts its life with a large pool of amino acids, but a peculiar one because of its abnormal composition relative to that of typical proteins. A very large fraction of the osmolarity of the sea urchin egg is provided by glycine. Some of the other amino acids, such as leucine, are in short supply in the pool. In any case, there is such a pool, but I would guess that it is probably not adequate for prolonged synthesis of a variety of proteins, such as begins at the beginning of development. Now, I cannot tell you what the real rate of protein synthesis is following fertilization, because we don't have proper information about the pool changes. The total protein of the egg does fall by about 15% from fertilization until the larval stage. However, the egg has in it a very large amount of yolk, most of which is gone by the end of the larval period. This yolk is mostly protein, 90% or so. Consequently, there is a very significant transformation of protein. There must be a lot of traffic through the pool, most of it being provided by yolk at one end and stable new proteins at the other.

KOHNE: May I make a comment on the first question? In these embryos, it is very difficult to do quantative studies and it would be very difficult to determine turnover if it were occurring.

GROSS: Yes, most of what I said is perhaps circumlocution. We don't have adequate pool data, nor suitable "chase" techniques for obtaining a satisfactory answer to the question.

POLLARD: The protein turnover with respect to yolk wasn't circumlocution, was it?

GROSS: No. It is clear that some protein disappears and new protein forms. Perhaps in the course of the discussion, we can come back to yolk.

GRUN: Is there an obvious simple explanation for this inverse relationship between the specific activity curves and the OD curves shown in Fig. 4?

GROSS: Suppose that the counts in each fraction were invariant, so that the computation of a specific activity involved a division at each gradient point of a constant number by a variable. The variable number, i.e., the optical density, is alternately high and low. Where it is low, you get a high value of specific activity, and where it is high, you get a low value. Therefore, in such an ideal case, with radioactivity a straight line of zero slope throughout the gradient, the computed radioactivity would be a pattern exactly the inverse of the optical densities. Now, as the actual radioactivities deviate from that ideal condition, the oscillations in the computed specific activity will be damped, and when the optical density and radioactivity are completely coincident, the computed specific activity becomes a straight line. I'm about to raise this point again in connection with radioactivity in the unfertilized egg.

HYMER: May I ask about the high specific activity in the region of the gradient containing molecules larger than 28S? Is there evidence for a heavy ribosomal precursor molecule in your system?

GROSS: No, there isn't. Certainly not at the beginning of development when there isn't any evidence of accumulating ribosomal RNA.

KOHNE: A "heavy" ribosomal precursor has been demonstrated in *Xenopus laevis*.

MASSARO: I'd like to deviate from the subject for a second. During the early protein synthesis, what contribution is the male component making? From this type of analysis, we're developing a type of parthenogenetic embryo.

GROSS: There is no difference in the pattern of protein synthesis - at least that we can determine by methods that I'll discuss later - between a parthenogenetic merogone, which has neither a sperm nucleus nor an egg nucleus, and the fertilized egg during the first few cleavages.

KOHNE: What is very interesting is that such an active process could go on at the low levels of sRNA present in eggs.

GROSS: It's a maximum of 5% in the sea urchin.

POLLARD: It is also interesting that this increase in sRNA seems to be in response to a need, because breaking down the yolk protein gives a large supply of amino acids, and this needs transfer RNA in order to be used, also.

GROSS: Yes, and that suggests, of course, that putting the CCA on has something to do with protein synthesis.

POLLARD: Well, a much higher concentration of tRNA will be necessary anyway, and whether the CCA addition is an essential part of that process or not I don't know. The concentration of transfer RNA has to be raised in order to actually get the amino acids in the proper location on the template. Actually, it's almost certain in a cell which is fairly big like this that the numbers have to be raised very considerably unless there is compartmentation, and protein synthesis occurs only a small regions. Still, I don't think that answers your question. You want to know why the sudden burst in the CCA part of it occurs.

GROSS: Yes, there is no question that there is a rapid net synthesis of sRNA when the protein synthesis rates rise for a second time at gastrulation. There begins a rapid internal synthesis of sRNA at that point and that is quite reasonable. However, why the entire CCA triplet should be knocked off and put back on, I don't know. At least I know of no evidence indicating that such an event is necessary in order for the sRNA to transfer and activate amino acid.

DEERING: Does anyone know whether this end group is actually present during the earlier stages or is it just added later?

GROSS: There is some uncertainty about this, but some evidence that has recently become available indicates that functional RNA is present in the unfertilized egg, that is, sRNA with its CCA triplet intact.

PAPACONSTANTINOU: Did your base ratio for that RNA give a GC content of 78%?

GROSS: No, but that is just the base composition of the new RNA, determined from hydrolysis of the bulk. It gives information only about what bases are being incorporated. It is possible that they are being added to populations of sRNA molecules that don't have any CCA on them at all.

PAPACONSTANTINOU: Then how could you test those for a P^{32} base ratio of 78%?

GROSS: Think of a piece of RNA in the presence of medium containing radio-phosphate. Then add to it pC, pC and pA, all of them radioactive. These are added to base X on every molecule. Now, stop the reaction, purify the material, and hydrolyze it. One residue comes off with no phosphate because the chain runs in the wrong direction. The next comes off with the phosphate and then X comes off with the radioactive phosphate. Nothing has been labeled at the next location. Radioactive XP can be any one of the four bases. Let's assume for the moment randomly so. That is what the base composition implies. Measuring the radioactivity of the phosphate, the composition of the material I have labeled in the way shown here, will be determined at 75% C and the rest distributed among A, U and G.

PAPACONSTANTINOU: I still can't see how you can explain a 70% GC unless you have an active CC turnover. That's the only way you can explain it. You have evidence that there is CCA present in the early embryo because of the 78% GC. However, suppose you started out with all the sRNA having no CCA on it. Would you still get this pattern?

GROSS: Yes.

As to the RNA of the unfertilized egg, there is strong but indirect evidence that the templates carrying the information for most or all of the protein synthesis that occurs during the period of cleavage are already present in the unfertilized egg. If that's so, then one is dealing with RNA templates that are storable under conditions of non-use and are very stable when they do begin to be used. In the presence of actinomycin, in doses sufficient to shut off new RNA synthesis, the primitive pattern of protein synthesis persists for a long time. It is a matter of some interest, therefore, to attempt to demonstrate directly that such a maternal messenger fraction exists, and second, to isolate it. It would be useful to isolate it because whatever approach works for the isolation would surely tell us something about the state of this material in the cell, and that, in turn, might tell us something about the control of its translation.

Nothing has been done as yet about isolating this material in bulk. A number of steps have been taken, however, to demonstrate its existence more directly. One approach is to make the RNA of an unfertilized egg radioactive during oogenesis in order to show that among the radioactive species there are some that are not ribosomal or transfer RNA. This has been done with most success in the amphibian (e.g., 9), and I'll leave it for Dr. Kohne to discuss. It has been possible to do the same sort of

thing in sea urchin eggs under more trying biological circumstances. If radioactive RNA precursors are injected into a gravid female sea urchin, no radioactivity is found in the mature eggs. This indicates that mature eggs are finished and no longer making any RNA and none can be forced in when the female is ready to spawn. An alternative is to make a female spawn and then let her carry out oogenesis, making a new crop of eggs in the presence of radioactivity. This is not a very practical procedure, at least with the species available to us, because it means having animals in large tanks of sea water, containing high levels of radiophosphate circulating in the sea water for weeks. However, there is a simple trick that can be done. This is to make a female spawn partially at the height of the normal reproductive season and then to place her in a tank that contains radioactive precursors for about a week. Under those conditions (2), a few of the oocytes complete their maturation to replace the ones lost in the partial spawning. We collect the mature eggs. Some of them are highly radioactive, as I'll show you, and in those the distribution of radioactive RNA can be studied.

Figure 6 is a section of an ovary of a sea urchin. This is a highly lobulate organ, whose walls contain an epithelium that gives rise to the ootids. There are oocytes in this wall in all stages of development, and an oocyte is identifiable by its large germinal vesicle nucleus. In some cases, you can see a nucleolus. This is prominent because the oocytes are growing and making ribosomes very rapidly. The ultimate product of the differentiation - and I use that word advisedly - of an oocyte into an egg is an ootid. It is recognizable here by its small pronucleus. These ootids fill the central parts of the lumina of the lobes. When the animals spawn, there is a highly stretched muscle in the outer layer of the ovary that contracts and fully mature ootids are extruded while the small immature eggs remain inside.

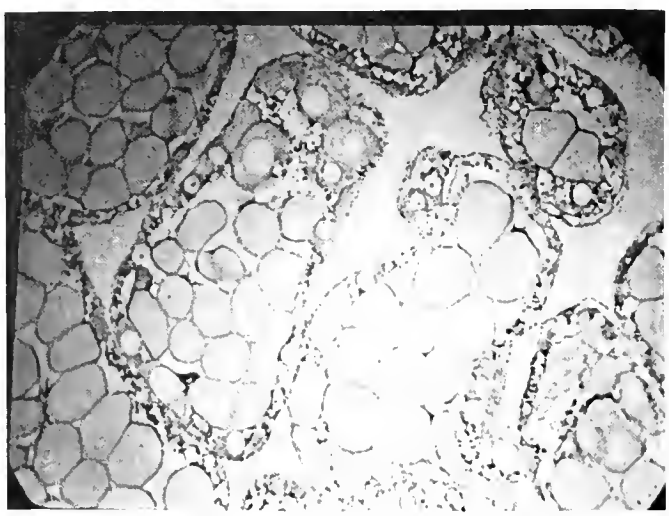

Fig. 6.

Now, if you perform the trick that I have described - partial spawning and labeling for a week - you find that it is possible to force radioactivity into the cells as represented in the autoradiogram shown in Fig. 7. This shows a region near the wall. The wall consists of three layers, an outer and an inner one, and a muscle layer. The oocyte layer is next to the wall. You see all of these cells are highly labeled, both in nuclei and in the cytoplasm, after a week. At the top of the figure is the luminad region with the cells getting larger. Everything in the region of the wall is radioactive, except for one cell that happens to be outside the wall and was fixed.

There is an interesting progression, as shown in Fig. 8. Close to the region shown in Fig. 7 about three-fourths of the cells are labeled, indicated by the left part of Fig. 8. When they are, the number of silver grains over each one is about the same. Those that are not labeled have no counts above background. There seem to be very few intermediate conditions of radioactivity between cells that have been making RNA at some constant rate during the time of exposure and the unlabeled ones that have finished before the radioactivity was supplied. Moving toward the lumen (left to right in Fig. 8), the number of labeled cells becomes smaller until finally in the central lumen, where the oldest eggs are, there is no label at all. This fact suggests that we are causing a few eggs to complete their maturation and labeling them while this is in progress. Silver grains represent counts in RNA, because all these sections are DNAse treated.

We can extract and purify the labeled RNA. The pattern obtained is shown in Fig. 9. One thing is at once apparent. During the time that labeling took place, these eggs were making all the bulk kinds of RNA. Both ribosomal species and 4S become radioactive and the radioactivity (faint solid line) and bulk patterns (dotted line) are superficially coincident. Therefore, these eggs, during the late stages of their maturation, are still making ribosomal RNA and presumably ribosomal proteins as well.

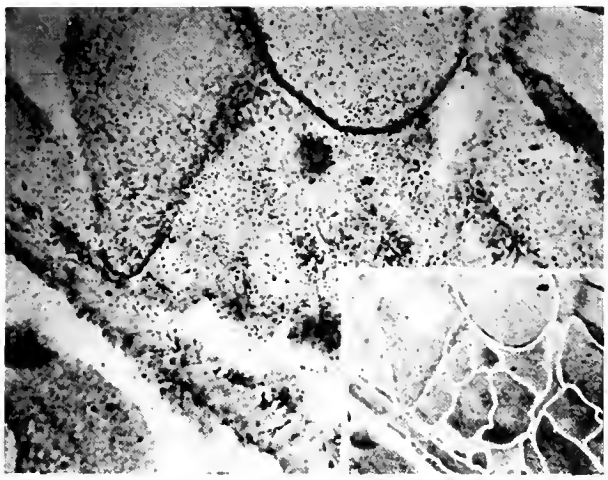

Fig. 7.

(Plate II, Gross, Malkin and Hubbard, *J. Mol. Biol. 13*, 463, 1965; reproduced with permission of Academic Press.)

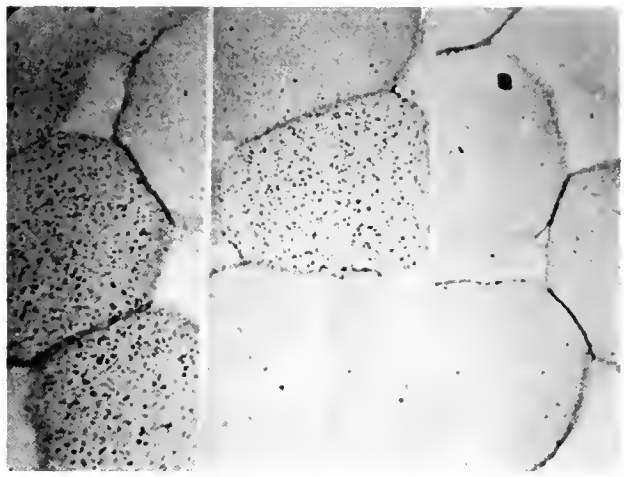

Fig. 8.

(Plate III, Gross, Malkin and Hubbard, *J. Mol. Biol. 13*, 463, 1965; reproduced with permission of Academic Press.)

They appear to be assembling complete ribosomes up to the very end of oogenesis. Now it is interesting that when one plots specific activities, determined after careful registration of counts and optical density for each fraction, one gets the sort of pattern shown by the heavy line in Fig. 9. There are several things that could give rise to deviations from constancy of specific activity in the manner shown. If the counts really represent what's present in bulk, then, of course, there should be no deviations from constancy. Now one possibility is that some extra counts are present throughout the gradient; i.e., that there is not complete coincidence between the optical density and the radioactivity. In that case, the pattern obtained will be of the type with maxima at the positions of the optical density minima.

There are two other possibilities, both of them representing technical errors: (a) that some highly radioactive bacterial RNA is present as a contaminant which would sediment slightly out of coincidence with the sea urchin RNA because the sea urchin species sediment at 18 and 28S, whereas the bacterial RNA sediment at 16 and 23S. On the other hand (b), perhaps for some unknown technical reason, we've failed to register the counts and optical densities accurately. In neither case would the pattern of deviation from constancy of specific activity be what is observed. A simple periodic function ratio shows that the pattern obtained would be one of constantly varying deviations across the peak, but no minima under the peak of optical density. These functional points are, however, less important than the fact that constancy of specific activity across the ribosomal density peaks is in fact obtained when the RNA is labeled late in development at a time when ribosomal RNA synthesis predominates. This was demonstrated on an earlier slide. Since these materials are treated and analyzed in the same way as those obtained from the labeled unfertilized eggs, there seems to be no doubt

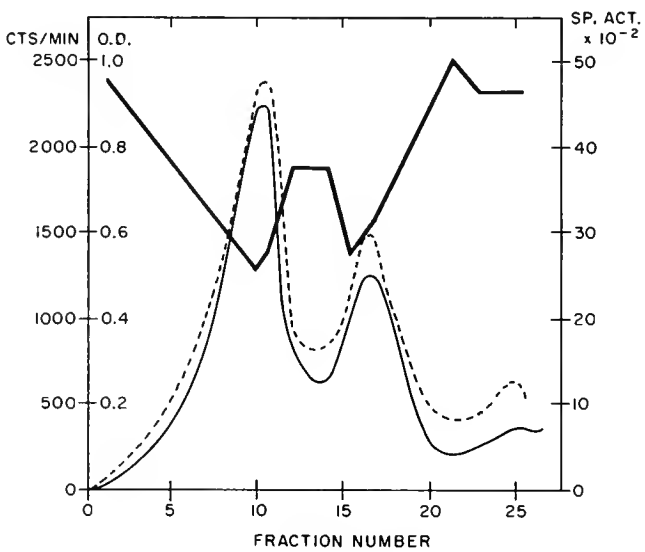

Fig. 9.

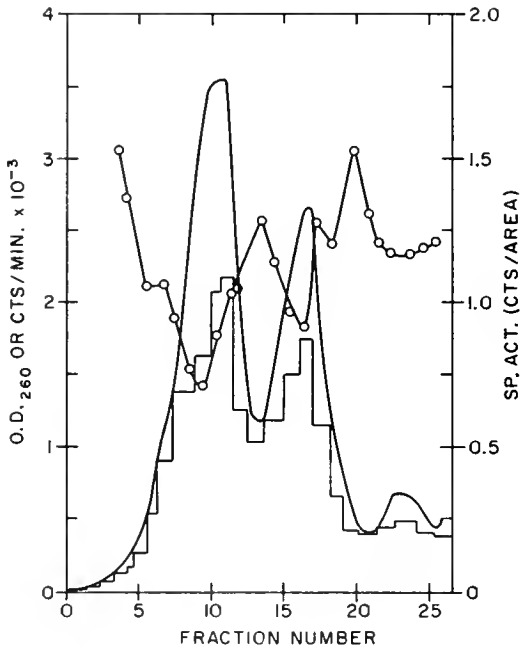

Fig. 10.

(Fig. 1, Gross, Malkin and Hubbard, *J. Mol. Biol. 13*, 463, 1965, reproduced with permission of Academic Press.)

that the deviations from constancy of specific activity do represent the first condition, that is, the presence of a small amount of RNA of high specific activity, not coincident with the ribosomal species. From the sizes of the specific activity variations, one can make a crude estimate of the amount of heterogeneous radioactivity. There is no good theoretical way for making such an estimate, but it is possible to make simple models composed of Gaussian error curves to represent the bulk species and extra counts distributed in roughly the way one might expect heterogeneous RNA to be distributed. You see in Fig. 10 that the order of maximum deviation of specific activity from unity is two (circles). Figure 10 shows a real gradient of specific activity. The reason that we drew the optical density curves (smooth curve, solid) continuously is that this is how they emerge from the Gilford recorder. We do, however, in each case, select individual fractions, measure their optical densities again, and then count them so that the specific activity as plotted results from the division of an actually measured optical density by an actually measured count.

With the model shown in Fig. 11, which is

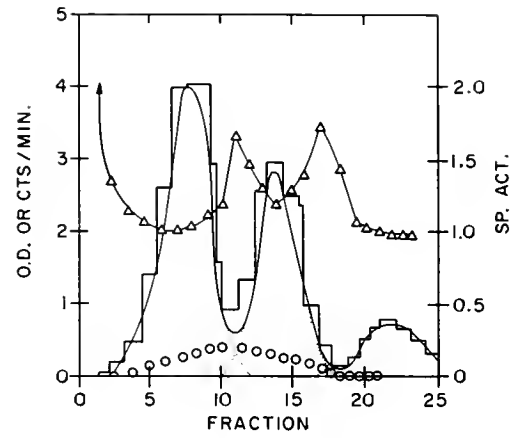

Fig. 11.

the closest one that we've been able to construct to the experimental results, there are 15% extra counts (large circles) distributed heterogeneously among the total in these preparations. (Specific activity, triangles).

There's only one final objection to this, and it is another kind of technical error. There might be absorption, or simply quenching, that results from the presence of RNA in these samples, and the amount of quenching could therefore be directly proportional to the amount of RNA. This has been checked, and it is not so. The specific activity deviations are therefore real and, on the basis of the model, they result from the presence of some 10 to 15% extra radioactivity in these preparations, sedimenting out of coincidence with the ribosomal and transfer RNA's. The suggestion is, therefore, that this is the messenger RNA in the unfertilized egg.

UNKNOWN DISCUSSANT: Let me ask you a technical question. What label were you using in these studies?

GROSS: The first one, without data points (Fig. 9), was labeled with P^{32}; the second one (Fig. 10) was labeled with uridine.

UNKNOWN DISCUSSANT: And did you use DNA digestion to eliminate any possibility of DNA labeling?

GROSS: Yes, DNase digestions are done routinely. There are a number of alternative possibilities for checking the conclusion that this represents messenger RNA. One is to examine the hybridizability of the radioactive RNA with DNA. We've done this, and it is by no means an easy thing to do because the specific activities of these preparations are

quite low. However, only a small fraction of the hybridizable radioactivity in preparations like this is ribosomal RNA. From competition experiments, we get a crude estimate of the fraction of the genome that's involved in the synthesis of ribosomal RNA and that appears to be about 0.2%. Most of the counts that do hybridize appear to be attached to sites on the DNA for which ribosomal RNA does not compete. I might say finally that unless one is familiar with this field, one might tend to be impressed with the result just described. However, there are, in principle, much better ways of doing it. The best, by far, seems at the moment to be an experiment showing that in the unfertilized egg there is a kind of RNA capable of supporting protein synthesis *in vitro*, an RNA other than degraded ribosomal or transfer RNA, and if one obtains an *in vitro* system that demonstrates this in a reliable way, then the problem has really been solved properly. There is a result from Monroy's laboratory (10) that appeared about a year ago, showing this to be the case, although the total incorporated activities were quite low. Nevertheless, their claim, and it seems to be a justified one, was that there is as much template RNA in an unfertilized egg as there is in an early blastula. That is certainly in accord with the indirect evidence described earlier.

We come now to the final point, which concerns proteins. First, there is some reason to suspect that among the proteins made at the beginning of development, are some that must be important for mitosis. Inhibitors of proteins synthesis, such as puromycin, also inhibit cleavage (11). They inhibit all of development, of course, but they do stop an ongoing cleavage if applied before metaphase. These inhibitors therefore stop division at a characteristic cytologic stage - a stage just before the mitotic spindle is formed and the nuclear membrane breaks down. All of this suggests that there are among the early proteins some that have something to do with mitosis. Autoradiograms of eggs labeled with amino acids make this suggestion in another way. We make such autoradiograms as a control whenever we label sea urchin eggs. The reason for this is that when dealing with animal cells, in a medium like sea water, the problem of bacterial contamination is ever present. One way that one can be reasonably sure that the radioactivity being studied is really inside the cells is to make autoradiograms and, hence, we do so routinely.

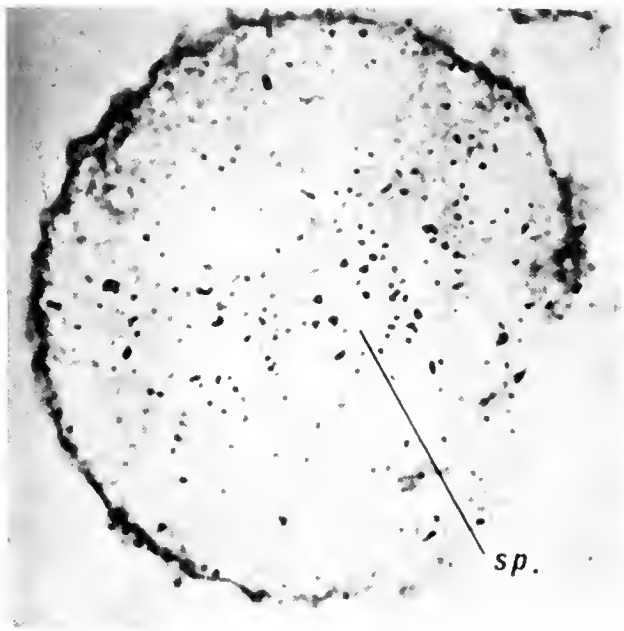

Fig. 12.

(Fig. 7, Gross, Malkin and Hubbard, *J. Mol. Biol. 13*, 463, 1965; reproduced with permission of Academic Press.)

Examining autoradiograms of cells that have been labeled with amino acids, during the first division cycle, we observe the sort of thing shown in Fig. 12. In cells that were at metaphase or early anaphase, there was a heavy concentration and, indeed, an almost exclusive localization of radioactivity in the mitotic spindle. Shown in the figure is an early anaphase mitotic apparatus. Now there are two possible interpretations of this result, and the one you accept depends on your hypothesis of the organization of the mitotic apparatus. If you believe that the mitotic spindle as seen *in situ* is a simple structure, most or all of whose protein is uniquely characteristic of it, then an autoradiogram of the type shown proves that most of the radioactivity goes into one protein, i.e., that all protein synthesis at the beginning of development has to do with the mitotic apparatus. The alternative arises if you don't believe that the spindle has in it only spindle proteins, but that it may have others as well. Then you have to decide whether the localization may mean something else. Figure 13, which is an electronmicrograph, shows why it is our conviction that the second alternative has to be accepted. This is a section through an early anaphase spindle at moderate magnification, and it is

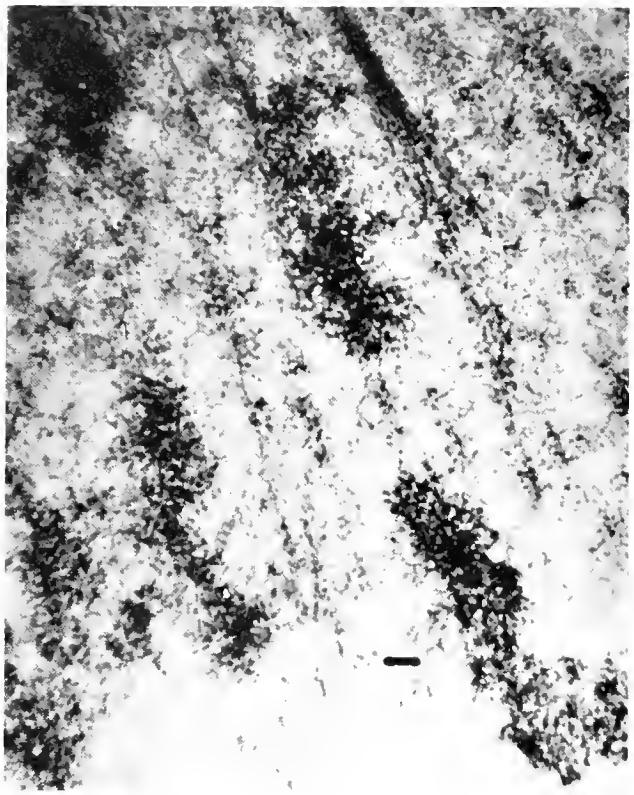

Fig. 13.

meant to show the spindle fibres which in the best preparations occupy, as you can see, a rather small portion of the total area or volume of the spindle. There are also chromosomes and background or matrix material. This matrix is very densely populated with vesicles, fragments of membranes and a large number of particles. The particles are of the same size and of the same electron density as are the ribosomes seen elsewhere in the cell, and there is no reason to believe that they are not ribosomes. If the characteristic spindle protein is what makes these fibres, then one would conclude from a picture like this that most of the protein in the spindle is not the characteristic microtubular protein. It is ribosomal and soluble protein.

A second consideration is relevant. If we were to take a sample volume in an egg without an organelle, like the mitotic spindle, we would find that there were a certain number of yolk particles in that volume. These yolk particles are solid objects. They don't seem to have the high degree of crystalline order in sea urchin yolk that's seen in some other species, but the particles are nevertheless very dense and have a high protein content. If the spindle or an organelle like it is formed, the yolk particles are extruded and indeed one can see large particulates such as yolk and mitochondria extruded from the forming spindle. Hence, the mitotic apparatus has in it no particles of the size of yolk and mitochondria. Soluble proteins, on the other hand, are presumably not extruded from the forming mitotic apparatus, because ribosomes are not, and the soluble proteins are smaller. Thus, if one were to measure the concentration of soluble proteins in the mitotic apparatus, and in the region outside of it, one would certainly find that the concentration of soluble proteins is higher within the region of the mitotic apparatus than it is in the periphery, simply because the peripheral material has in every volume element a large excluded subelement occupied by the yolk. On this basis alone, any report that something is localized in the spindle should be viewed with caution. For example, there are reports in the literature on the cytochemical localization of enzymes and certain thiol-rich proteins in the mitotic apparatus, but I would venture to predict on the basis of the argument just given that at least some of the observed cytochemical localizations are localizations by default and not the result of active processes associated with the assembly of the mitotic apparatus. I wanted to make this argument clear because it suggests that the radioactivity seen in the spindle may have been included in that region in a passive rather than an active fashion. One possibility exists for testing this question further, and that depends on the presence in the spindle of fibres or microtubules that presumably represent the definitive working part of the organelle.

Figure 14 is an optical autoradiogram of an isolated spindle sectioned at one micron. This spindle is a member of a population obtained from eggs that have been pulsed with the amino acid leucine and "chased" prior to the appearance of the metaphase spindle. You might already see in the figure a suggestion that the radioactivity, which is represented by the silver grains, has a certain tendency to follow the lines of the fibres. These fibres, which run in tracts, are visible in sections of this thickness. Figure 15 is an electron microscope autoradiogram made from the same material. At low magnification, one sees tracts of fibres running through the center of the spindle and silver grains distributed over the whole area.

Flg. 14.

(Fig. 1. Mangan, Mlki-Noumura and Gross, *Science 147*, 1575, 1965; copyright 1965 by the Association for the Advancement of Science.)

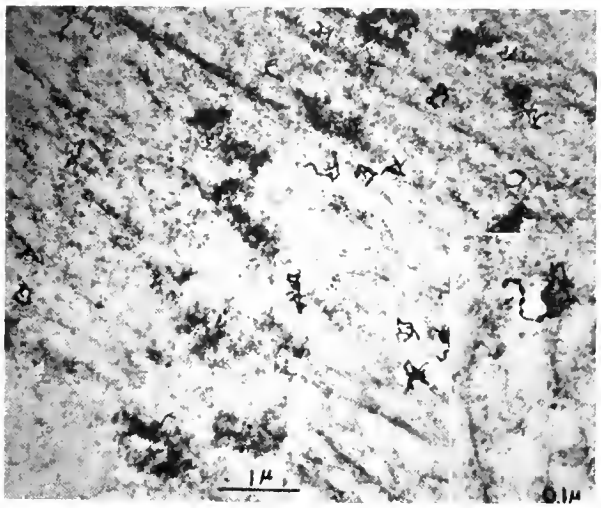

Fig. 15.

Now one's impression is certainly that a very large fraction of these silver grains are either on or next to the fibres. Does this mean that the fibres are labeled? I think that it does, for the following reason: either the fibres are more radioactive than the region as a whole, or they are not and the radioactivity is simply randomly distributed. There are a number of ways to test such a question, and the next two figures show the way that we elected to do so. A sheet of acetate overlay is placed on a print of the type shown in Fig. 15. A circle, whose diameter represents the average silver-grain diameter, is drawn on the overlay over every grain, and wherever a fibre occurs next to or under such a grain, the fibre is indicated and that grain scored as a hit. Figure 16 is the overlay pattern for the print shown in Fib. 15. Next, the area of the print is divided into a large number of coordinates, say 10,000, and then using these coordinates and the total number of silver grains in the actual print, a number of points is selected from a table of random numbers equal to the number of grains. These points will, of course, be randomly distributed over the coordinate grid. Circles representing the points selected from the random number table are drawn on a new sheet of overlay, placed over the print, and a fibre is scored as a hit when it is adjacent to or under one of the circles.

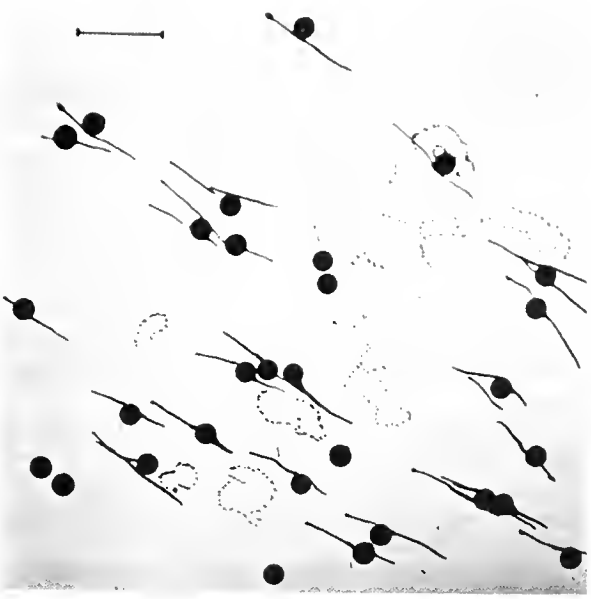

Fig. 16.

The result is shown in Fig. 17. Now, it is always observed that the number of hits obtained with randomly-placed points is much smaller than it is with the actual prints and grain patterns. This rather rigorous test suggests, therefore, that the microtubules are in fact labeled. There is radioactivity in the interstices, as one might have expected, but a large fraction of the radioactivity, a larger fraction than would be expected on the basis of chance alone,

Fig. 17.

appears actually to be on the fibres. Therefore, one of the first proteins synthesized in the early development of the sea urchin and presumably one of those for which the program is stored in the egg prior to fertilization, is a protein that has some function in the organization or operation of the mitotic apparatus.

I believe that my time is up and we will therefore have to defer a discussion of other products of early protein synthesis to another occasion.

POLLARD: Thank you very much. Are there any questions for Dr. Gross?

CHALKLEY: Do the ribosomes from the mature egg support protein synthesis under *in vitro* conditions?

GROSS: There has been some argument about whether unfertilized ribosomes are competent to support protein synthesis. An alternative explanation to the maternal messenger story might be that there is a lesion in the ribosomes of unfertilized eggs which is healed on fertilization. That is indeed a part, at least, of the point of view of Monroy and his collaborators (12). Nemer (13), on the other hand, has presented what was, I believe, reasonably good evidence that ribosomes from unfertilized eggs work well. In his experiments, they operate with poly-U and with other synthetic polynucleotides. Monroy explains that the ribosomes from fertilized eggs respond well to natural messages, while the ribosomes from unfertilized eggs do not. The point of their recent paper is that unfertilized ribosomes which respond very poorly to natural messengers *in vitro* can be made to respond normally by a brief treatment with trypsin. They are suggesting that the unfertilized ribosomes are blocked, perhaps with a protein, and that one of the first events of early development is the removal of that block, possibly by proteolysis. It should be pointed out, however, that the same group of investigators have shown that in this material endogenous mRNA levels are about the same in unfertilized eggs and blastulae.

DEERING: Do you know what happens to the RNA situation when you artificially activate an egg?

GROSS: If you do this successfully, you turn on both protein and RNA synthesis in the normal way, since one gets a normal haploid embryo.

MAURER: What about nuclease activity? Could it be that the stability of your messenger is due to a low level of ribonuclease?

GROSS: It could, but it is certainly not so. These eggs have extremely high levels of nuclease, so that the problems of handling the RNA are very complicated, indeed.

MAURER: Can you inhibit by bentonite?

GROSS: Yes. You can inhibit the nuclease activities sufficiently to make what look like respectable RNA preparations, but this does require rather heroic efforts. There is only one way I know of dealing with the high levels of nuclease when such activity must be stopped entirely. We learned of the trick when working with polyribosomes. This is to add either large amounts of enucleate HeLa cells, that is to say, HeLa cell cytoplasm, or large amounts of yeast RNA. In both cases, what one is doing is providing the endogenous nucleases with a large excess of substrate in the hope that the substrate in which one is interested will remain, to a large extent, untouched.

CHALKLEY: Wouldn't this raise a very interesting point, then? First, you have a very stable RNA in the cell and a lot of nuclease present and, presumably, not able to attack and disrupt it; later the problem arises that it can attack it. One might think of compartmentation playing a role.

GROSS: Yes, I believe it would be a necessary conclusion. If the nuclease is really there,

then either the RNA or the nuclease is sequestered.

CHALKLEY: Then the point I'm aiming at is that the RNA is not in some mysterious way stabilized.

GROSS: It's not easy to distinguish at this point between the two proposals.

HYMER: I would like to comment on this point. Dr. E. L. Kuff and I demonstrated the presence of an endonuclease within nuclei isolated from murine plasma cell tumors. This enzyme preferentially attacked rapidly labeled high molecular weight RNA, and its activity could be completely inhibited by the addition of cytoplasmic soluble fraction.

GROSS: Well, in any case, the whole problem of stability and instability in messages is both interesting and difficult, and it is by no means restricted to embryos. On the basis of a large body of accumulating evidence, one can now safely conclude that stable and unstable messages coexist in the cells of higher organisms.

UNKNOWN DISCUSSANT: You mention that you are able to hybridize the nucleic acid from the unfertilized egg. What percentage of hybridization were you getting and what technique were you using?

GROSS: Our technique was a modification of the Nygaard-Hall method, essentially the one described by McConkey and Hopkins in the *Proceedings of the National Academy of Science* about a year ago (14). The method gives low values of hybridization. In fact, McConkey and Hopkins got a value for the size of the ribosomal fraction that is obviously much too low. Their method has the one virtue that it reduces so-called mistaken identity hybrids to the lowest values that I know without the use of ribonuclease. We use this method, therefore, because our low specific activities and large amounts of ribosomal RNA demanded it. With it, we get something like 1-1/2% hybridization. That is 1-1/2% of the total counts in a preparation of the type for which we saw gradients earlier, hybridized under the conditions of saturation routinely employed. By using a 5 to 15-fold excess of unlabeled RNA, we can reduce the counts by only a very small amount - 8 or 10% of the original number. From that reduction, we got the estimate of the fraction of the genome occupied by the ribosomal cistrons.

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EARLY BIOCHEMICAL EVENTS FOLLOWING FERTILIZATION OF SEA URCHIN EGGS[1]

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INTRODUCTION

Fertilization results in a metabolic activation, similar in certain respects to the activations occurring upon neurochemical stimulation of muscle or addition of hormone to target tissue. It differs from the above, however, in that fertilization occurs only once during the lifetime of the organism, initiating a unique series of reactions leading to rapid cell divisions and embryonic differentiation.

The changes which occur upon fertilization are dramatic at both the morphological and molecular levels. Changes in membrane structure, respiration rate, and rates of DNA, RNA, and protein synthesis occur, as well as changes in cation and coenzyme content, and subcellular location of enzymes. These all occur within seconds or minutes of insemination, and somehow are interrelated with each other to yield an orderly pattern of embryonic development.

Although many post-fertilization changes have been observed, numerous unresolved problems still exist. Little is known about how these changes occur, when they occur, or the casual connections between them. For example, it is not known whether synchronous activation of all enzymes is the case, or whether one or several changes are triggered which then initiate the other reactions in a chain or cascade-type reaction system.

The research to be discussed represents the beginnings of an intensive study of the fertilization reactions, aimed at shedding some light on the above problems. The experimental approach used is based on the assumption that the fertilization changes result solely from enzymic activation. The pertinent evidence for this is, first, that eggs can be artificially activated (artificial parthenogenesis) to develop without sperm (1). This indicates that the sperm does not supply some missing enzyme or substrate to the egg, and hence implies that all materials necessary for development reside in the egg. The second piece of evidence is that eggs can be fertilized in the presence of concentrations of puromycin sufficient to inhibit the bulk of protein synthesis. Under such conditions, they will develop up to the first mitotic division (90 minutes after insemination in the eggs of *S. purpuratus) before* any arrest occurs (2). This result means that little or no *de novo* protein synthesis is required for the earliest reactions of development, such as pronuclear fusion or RNA synthesis. These two experiments indicate that the immediate changes of fertilization most probably result from activity of enzymes already present in the egg.

Enzymes and metabolic pathways activated by fertilization, as well as physicochemical changes possibly controlling these activations, are shown in Table I. This table categorizes the best described post-fertilization changes in sea urchin eggs as changes in carbohydrate and energy metabolism, co-factor and coenzyme metabolism, synthetic metabolism, and changes in structure.

Examination of these changes suggests some possible factors limiting metabolism in the unfertilized egg. For example, the metabolic machinery of the egg might be limited by cations (as evidenced by changes in Ca^{+2} or K^{+}), by

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[1] Supported by Public Health Service grant 5T1 GM2G277 and National Science Foundation grant GB-4206.

[2] Present address: Hopkins Marine Station, Pacific Grove, California.

TABLE I

Metabolic and Structural Changes Upon Fertilization of Sea Urchin Eggs:

		References
A.	Carbohydrates and Energy Metabolism	
	1. Respiration rate increase	41, 42 (review), 14, 6
	2. Increased pentose shunt activity	39, 40
	3. Increased content of glycolytic esters	29, 43
B.	Cofactor and Coenzyme Metabolism	
	1. TPNH increase	9, 12
	2. Free Ca^{+2} increase	34
	3. K^{+} increase	44
	4. PO_4^{-3} uptake increase	45, 46
C.	Synthetic Metabolism	
	1. Increased rates of protein synthesis	47-53, 3
	2. Increased rates of RNA synthesis	54-56
	3. Increased rate of lipid synthesis	57
D.	Structural and Physical Changes	
	1. Cortical granule breakdown	19, 58 (reviews)
	2. Changes in subcellular localization of enzymes	32, 33
	3. Fertilization acid excretion	59, 22
	4. Proteolytic activity increase	60
	5. Membrane potential	15, 16
	6. Light-scattering change in cortex	13

coenzymes (as evidenced by increased TPNH), by lack of respiratory substrate (as evidenced by increased content of glycolytic esters, respiration rate, etc.), by unavailability of substrate to enzyme (as evidence by both structural changes in cortex and intracellular location of enzymes, as well as the transient proteolytic activity), or possibly by presence of a general inhibitor (as suggested by acid excretion or proteolytic activity).

To decide between these alternatives, a kinetic analysis has been used and will be described in this paper. Such an analysis, aimed at describing the temporal sequence of the fertilization reactions, should yield information on possible mechanisms of activation. Hypotheses derived from the kinetic analysis can then be tested, hopefully leading to elucidation of any primary reaction or reaction series of fertilization. These studies should also provide rigorous testing of hypotheses. As an example, if the recent hypothesis relating proteolytic activity to the post-fertilization initiation of protein synthesis is correct (3), the transient activation of proteolytic activity should occur before the activation of protein synthesis.

To date, we have concentrated on the kinetics, mechanism, and metabolic significance of changes in coenzymes, carbohydrate and respiratory metabolism, acid excretion, and structural changes. The methods we have used measure *in vivo* changes in cell suspensions, using procedures developed at the Johnson Foundation of the University of Pennsylvania (4, 5). The basic equipment consists of a water-jacketted glass cuvette, into which is placed a concentrated suspension of eggs. From the side of this cuvette, optical measurement of light-

scattering (structural changes) and 366 $m\mu$ induced cell fluorescence can be made. This latter measurement, in all systems so far described, is specific for detecting changes in reduced pyridine nucleotide (4). Through the top of the cuvette can be inserted an oxygen electrode for measuring respiration rate, and a pH electrode for measuring excretion of the fertilization acid (see 6 and 12 for experimental details). Finally, samples can be taken from the cuvette for analysis of coenzymes, substrates, or enzyme activity. The four parameters (light-scattering, fluorescence, respiration, and acid excretion) have been monitored through low time constant amplifiers, and recorded individually on synchronized recorders, or simultaneously on a multi-channel recorder.

RESULTS

I. Temporal sequence of fertilization changes

A. Pyridine nucleotide changes

TPNH is the coenzyme generally involved in reductive biosynthesis, as indicated by the coenzyme specificity of reductive reactions, as well as by the general correlation between synthetic activity and both TPNH levels and TPNH/TPN ratios (7, 8). This compound has been reported to increase within one hour after fertilization (9), and hence this change might be important in initiating and controlling reductive biosynthesis in the egg.

As indicated, 366 mμ-induced cell fluorescence is a sensitive monitor of reduced pyridine nucleotide *in vivo*. Measurements of cell-fluorescence following fertilization, shown in Fig. 1, indicate an increase in this parameter, beginning at 40 seconds after sperm addition, and ending by 5 minutes with a 1/2 time of 35 seconds. Enzymatic analyses of reduced pyridine nucleotides in alkaline-extracted cell homogenates are shown in Fig. 2. These indicate that the reduced pyridine nucleotide which increases is TPNH, and that this increase parallels the changes in fluorescence. Furthermore, the sum of reduced pyridine nucleotides at various times after fertilization is linearly related to the cell fluorescence (Fig. 3), which confirms the relationship between *in vivo* fluorescence and reduced pyridine nucleotide.

The increase in TPNH does not result from reduction of pre-existing TPN, but rather from phosphorylation of DPN to TPN, and most probably the subsequent reduction of this TPN to TPNH. This is shown in Fig. 4 and Table II. Figure 4 shows that DPN decreases, while TPN increases in a mirror-image fashion. Similar behavior is also seen for the TPNH increase shown in Fig. 2. These changes suggest a precursor-product relationship, and this supposition is further verified by the stoichiometric relationship shown in Table II, which is a balance sheet of pyridine nucleotide before and after fertilization. The pertinent point to observe is that total amount of pyridine nucleotide is the same before and after fertilization, but that an interconversion of pyridine nucleotide types has occurred - total TPN and TPNH increasing, while total DPN and DPNH decrease. The enzyme implicated in such an interconversion is DPN kinase, which catalyzes the reaction:

$$\text{DPN and ATP} \longrightarrow \text{TPN and ADP (10, 11).}$$

This enzyme, then, is apparently activated by fertilization. Possible mechanisms of its activation will be described later.

POLLARD: How does that fit with any reasonable turnover numbers for the production

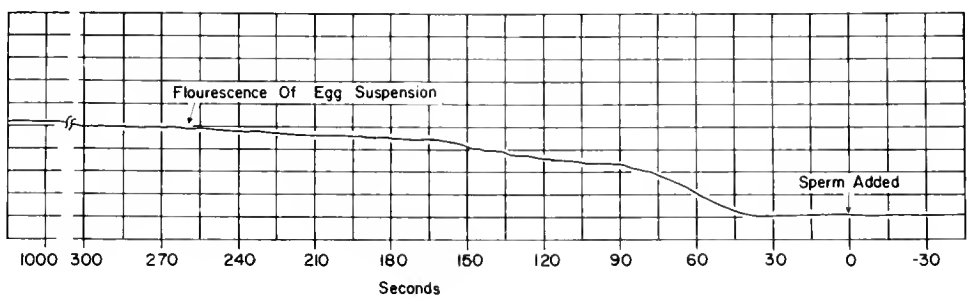

Fig. 1.

366 mμ induced fluorescence of eggs of *S. purpuratus* following fertilization. (Fig. 1, Epel, *Biochem. Biophys. Res. Comm. 17*, 69, 1964; reproduced with permission of Academic Press.)

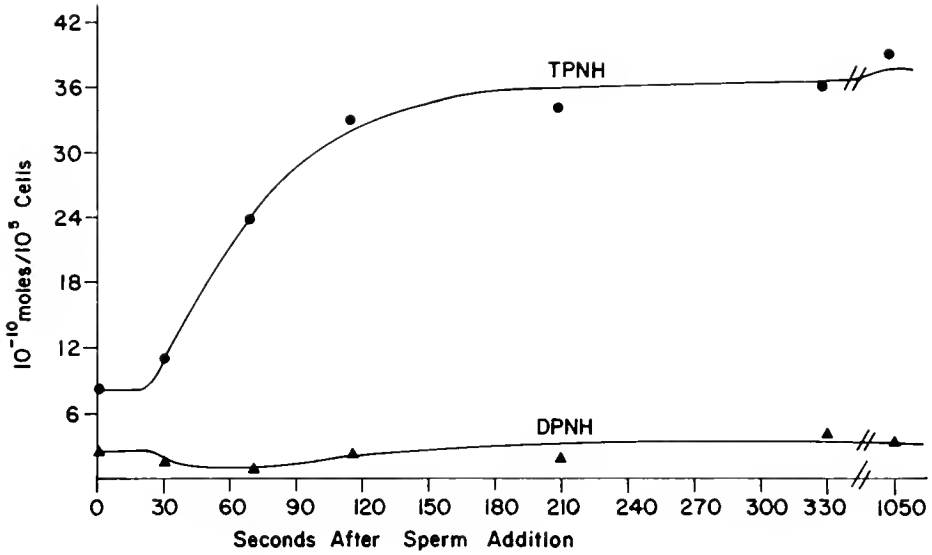

Fig. 2.

Analysis of reduced pyridine nucleotide at various times after fertilization of *S. purpuratus*. (Fig. 2, Epel, *Biochem. Biophys. Res. Comm. 17*, 69, 1964; reproduced with permission of Academic Press.)

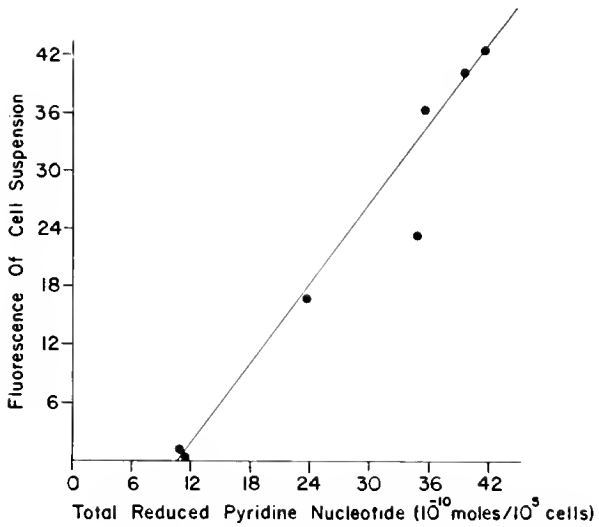

Fig. 3.

Linearity of cell fluorescence and reduced pyridine nucleotide at various times following fertilization.

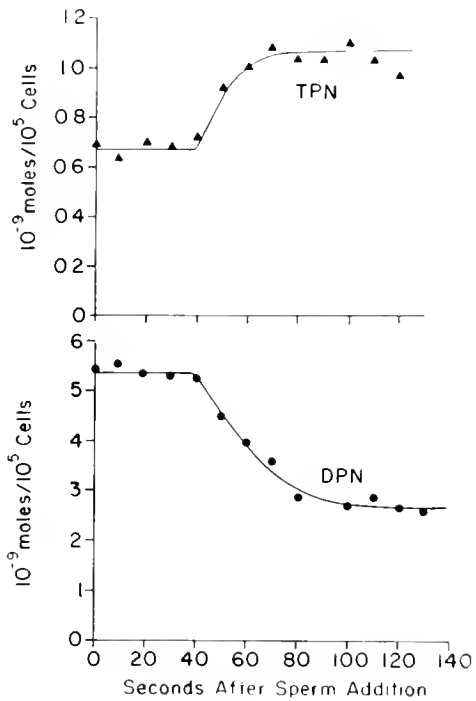

Fig. 4.

Analysis of oxidized pyridine nucleotide following fertilization of *S. purpuratus* (Figs. 1, 2 and 4 are from separate experiments and not strictly comparable).

TABLE II

Average Content and Ratios of Pyridine Nucleotides In *S. purpuratus*[a]

[a] 10^{-10} moles/10^5 cells

	Unfertilized	Ratio $\frac{TPNH}{TPN}$	Fertilized	Ratio $\frac{TPNH}{TPN}$
TPNH	6.7 ± 1.5	0.96	29.7 ± 9.0	2.8
TPN	7.0 ± 0.1		10.7 ± 3.0	
		Ratio $\frac{DPN}{DPNH}$		Ratio $\frac{DPN}{DPNH}$
DPN	59.3 ± 6.8	17.9	32.7 ± 8.3	6.6
DPNH	3.3 ± 2.5		5.0 ± 4.3	
Total	76.3		78.1	
% DPN & DPNH		82%		48%
% TPN & TPNH		18%		52%

of the TPN? You've got 10^9 molecules per cell formed in about 10 seconds. Isn't that quite rapid formation? Is there any "miracle" here?

EPEL: Any "miracle"?

POLLARD: I'm referring to the fact that 10^9 molecules per cell are made in 10 seconds.

EPEL: The data in the figure is per 100,000 cells.

POLLARD: It's 10^9 molecules per cell, which gives a really very rapid turnover number of about 10,000 per minute. Why are the enzymes that good? That would seem to me to be the exciting thing you've got here. Is it all right?

EPEL: Actually this is consistent with maximum activity of the enzyme. For some reason the enzyme is suddenly activated close to maximum activity, or at least within a factor of 2 or 4.

POLLARD: That is a slight miracle. Is it more than "maximum"?

EPEL: No, it's not more than maximum, as extrapolated from *in vitro* experiments under simulated *in vivo* conditions.

DEERING: This assumes you know how much of the enzyme is present.

EPEL: Yes. On the basis of extracting enzyme from a known amount of cells, and assaying kinase activity at ATP and DPN concentrations present *in vivo*. In any case, if it were grossly aberrant, we would notice it. This is the most active source of the enzyme that's ever been found. The maximum activity is only three times less than the 75-fold purified enzyme from pigeon liver.

B. Respiratory changes

Simultaneous measurement of respiration rate and cell fluorescence, shown in Fig. 5, indicates that the fluorescence change (TPNH increase) *precedes* the activation of respiration. Respiration is measured polarographically, and an upward deflection indicates a decrease in oxygen content. Rate is indicated by the slope. The respiration rate (see Fig. 7) is characterized by a transiently large burst, followed by a slow decrease to a rate 4-5 times that of the pre-fertilization rate. Significance of these kinetics, as well as possible controlling mechanisms for respiration, will be described later.

C. Excretion of the fertilization acid

Simultaneous measurements of fluorescence and extracellular pH indicate that changes in these two parameters began simultaneously if measured at similar amplification levels (i.e., at amplifications such that the total changes are of similar magnitude on the chart paper),

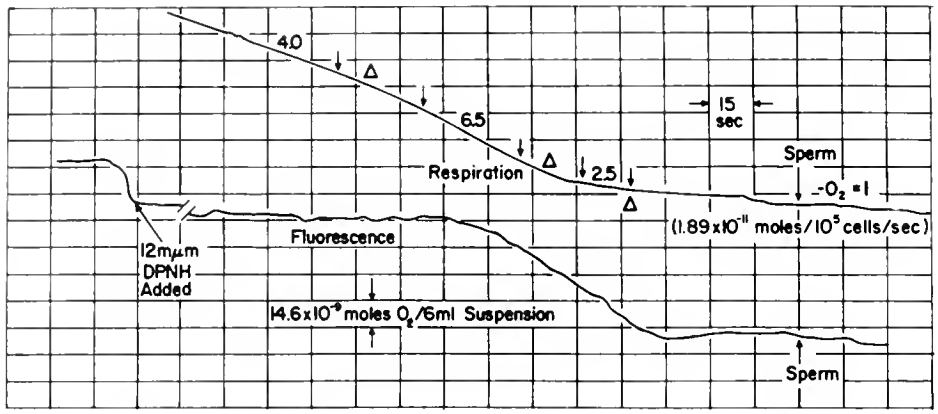

Fig. 5.

Simultaneous measurement of respiration and fluorescence following fertilization of *S. purpuratus*. Decrease in O_2 content is towards the top of the figure. Respiratory rates at various times are indicated on the trace, In 10^{-11} moles O_2 consumed/10^5 cells/sec. Time is from right to left.

as in Fig. 6. If measured at different amplifications, as in Fig. 8, the timings of the changes were apparently different, acid excretion preceding the fluorescence change.

The rate of the acid excretion, in eggs of all three species of sea urchin examined, always peaked before the peak respiratory rate (Fig. 7). This suggests that the reactions responsible for the acid formation occur very rapidly, and are essentially over before the respiratory increase. The source and mechanism of the acid formation will be discussed later.

D. Light-scattering changes

Light-scattering measurements can be a sensitive monitor of structural changes. Accordingly, the kinetics of light-scattering changes following fertilization were measured in collaboration with Dr. B. C. Pressman, using an instrument designed by Dr. Pressman (5). This instrument can simultaneously record all the parameters previously described.

The results of one such measurement are shown in Fig. 8. It is seen that a light-scattering decrease begins at 45 seconds, and is temporally coincident with the beginning of acid excretion. Within five seconds the fluorescence change begins, and this is followed at 60 seconds after sperm addition by the activation of respiration.

These measurements, then, indicate that a temporal differentiation of these events does occur following fertilization. In the remainder of this paper, I shall discuss first, the reality and universality of these kinetics, and second, the possible structural and molecular mechanisms of the observed changes.

II. Possible factors influencing the kinetic determination

Several questions can be raised as to the degree the observed temporal sequence reflects the actual sequence. A major biological artifact could be the kinetics of sperm-egg interaction. Thus, if the successful contact between egg and sperm took several seconds or minutes, the timing and duration of the observed changes could simply, and uninterestingly, represent the fertilization time. The experimental conditions which would obviate this argument, however, are (1) a large redundancy of sperm were added, and (2) the same kinetics were obtained in the presence of 10-fold less sperm.

The experimental measurements also provide an estimate of the time for successful sperm-egg interaction, which is related to the duration of that reaction completed in the shortest interval. From Fig. 8, this is seen to be the light-scattering change, which has a ½ time of only 20 seconds. The ½ time for fertilization is probably less than this, however, since the light-scattering change in a single cell probably has a finite duration. If this change is identical to that observed in single cells, its duration in one cell would be about 20 seconds (13).

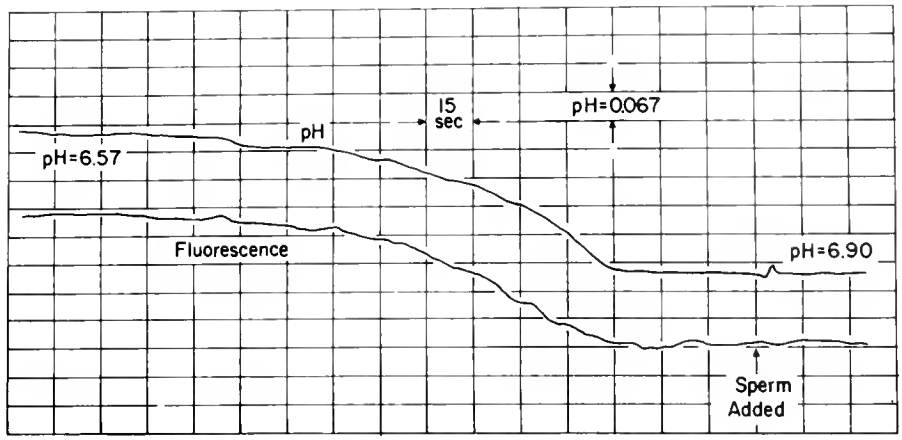

Fig. 6.

Simultaneous measurements of extracellular pH and cell fluorescence following fertilization of *S. purpuratus*. Note that time is from right to left.

The other question relates to whether the observed temporal sequence might result from instrumental artifacts. This is probably the case in the lag between the light-scattering-pH change and fluorescence change shown in Fig. 8. Thus, if the observed light-scattering or acidity changes were adjusted to give the same amplitude on the chart as the fluorescence change (as in Fig. 6), the temporal sequence would be almost identical (within two seconds). It is probable, therefore, that changes in acid excretion, light-scattering, and fluorescence all begin simultaneously, with possibly a slight lag in the fluorescence change.

The respiratory change, in all cases so far examined, always begins after the above changes and does not appear to result from any instrumental lag. First, when fluorescence and respiration rate are similarly amplified, the lag is still apparent. Secondly, when an

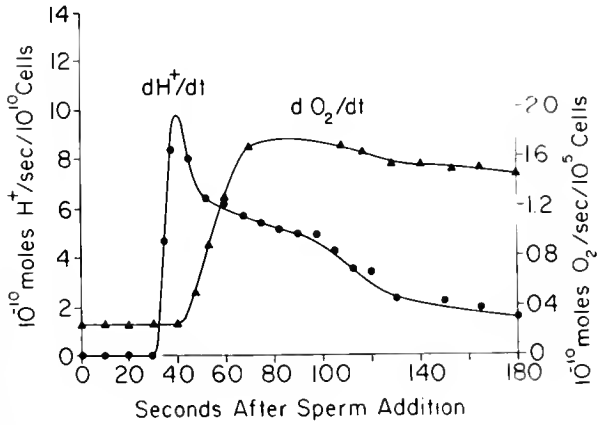

Fig. 7.

Derived rates of acid excretion and cell respiration following fertilization of *S. purpuratus*. Note that peak acid excretion occurs before the increase in respiratory rate.

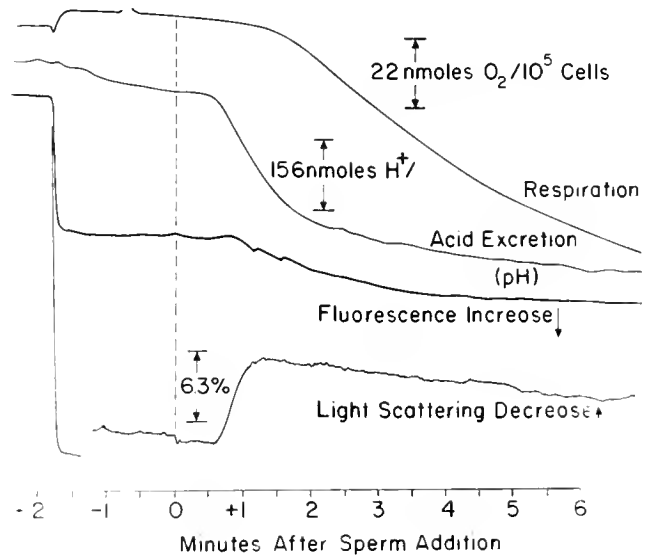

Fig. 8.

Simultaneous measurement of cell fluorescence, extracellular pH, respiration rate and light-scattering in eggs of *S. purpuratus*. (Data of Epel and Pressman).

uncoupler of respiration is added to fertilized eggs, there is only a 10-second lag before the increased respiratory rate is evidenced, as compared to a 30-second lag between fluorescence and respiration when these eggs were initially fertilized. Finally, the lag is evident in other species examined (see, e.g., Fig. 10), and was also observed by Ohnishi and Sugiyama (14) in several species of Japanese sea urchins. These workers, furthermore, were using a bare platinum electrode with time constants less than one second, as compared to our membrane-covered electrodes with time constants of 3-6 seconds.

The present data, then, indicate that the first discernable event of fertilization - in our measuring system - is a structural change, probably related to cortical granule breakdown (see Sec. IVa). This light-scattering change, observed in cell suspensions, is probably similar to that seen by Rothschild and Swann in single cells under dark field illumination (13).

Although this structural change occurs early, the first change in the eggs is undoubtedly related to attachment of the sperm acrosomal filament, which probably initiates these structural reactions in a primary, or possibly secondary, reaction. The structural events might also be related to changes in electrical properties of the membrane, as first shown by Tyler *et al* (15) and Hiramoto (16). The data of Hiramoto is shown in Fig. 9, and indicates an early change in membrane resistance, capacitance, and potential upon successful sperm-egg contact. This change precedes membrane elevation and might also precede cortical granule

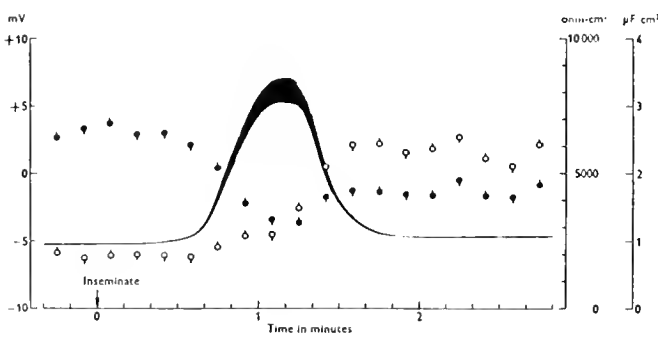

Fig. 9.

Data of Hiramoto, showing changes in membrane potential ♩, membrane resistance 𝅘𝅥, and membrane capacitance ♩ 𝄾, following fertilization of *Peronella*. (Fig. 2, Hiramoto, *Exp. Cell Res. 16*, 421, 1959; reproduced with permission of Academic Press.)

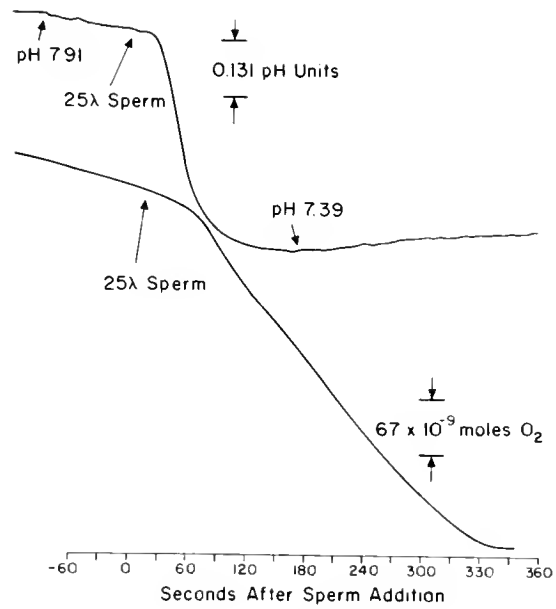

Fig. 10.

Respiration rate and extracellular pH following fertilization of *Lytechinus variegatus* (data of Epel and Iverson).

breakdown, although the temporal relationship between granule breakdown and membrane elevation is not clearly defined, and might vary in different species (17).

GROSS: The time is about a minute after fertilization, isn't that right?

EPEL: Yes.

MASSARO: Is that from the time of adding the sperm or from the time of contact?

EPEL: I believe it's from the time of sperm addition. However, the important point is that he shows data that indicate relative time of membrane elevation.

MASSARO: Well, how long does it take the sperm to get in? Where is the sperm after 15 seconds?

EPEL: That's a good question. In some or most organisms an acrosomal filament is ejected from the head of the sperm. In *Hydroides* this, supposedly, takes place within 9 seconds after you add the sperm.

POLLARD: Isn't that about where the first indication of change in membrane resistance is seen? The resistance shows quite a change right away.

EPEL: The best evidence for a rapid change is a change in light-scattering of single cells observed under dark field. This takes place

about 10 seconds after sperm-egg contact is made.

MASSARO: The sperm is on the outside with the acrosome penetrating?

EPEL: I don't think there is any direct evidence for that. Certainly the sperm head does penetrate within a short time. However, it takes a relatively long time for it to appear inside the egg.

GROSS: These things are all cortical changes?

EPEL: Yes. I doubt if the sperm is contributing anything in the initial chemical changes such as genetic information or enzymes getting inside the egg are concerned. As you indicated, the sperm doesn't get in until minutes after. These are surface reactions.

TS'O: These eggs can only be fertilized by a single sperm?

EPEL: You can get poly-spermy if you add a very large redundancy, but normally only one sperm penetrates.

GROSS: The barrier to poly-spermy takes about 20 to 45 seconds to develop at normal temperature. So you'd need a very large multiplicity.

EPEL: I think it's more like 10 seconds, although I wouldn't want to say it's that, definitely. [(Added in proof): A short note by Rothschild and Swann *(Exp. Cell Res., 2,* 137, 1951) indicates that the actual block to polyspermy takes at least 25 seconds, and probably longer. They interpret the failure of the kinetic calculation to apply to the *in vivo* situation as indicating that the limiting factor is the probability of a "successful" sperm-egg collision.]

There is one, so far unconfirmed, report which is completely revolutionary. This is a report by Neyfakh *et al.* (*Biochem. Biophys. Res. Comm. 18,* 582, 1965) on fertilization in fish eggs, which shows that simple contact with sperm is sufficient to activate synthesis of cytochrome oxidase. This activation occurs within one second, and is hence the most rapid change ever reported.

MAURER: Do we know anything we can do to the sperm which will eliminate this kind of surface contact?

GROSS: I don't know of any.

POLLARD: What happens if you ultraviolate the eggs and sperm *in vivo?*

EPEL: They're okay.

POLLARD: They still do it?

EPEL: Yes, you can chemically activate the egg without any sperm.

MAURER: What pushes the button in the sperm?

EPEL: Presumably interaction between sperm and egg result in ejection of the acrosomal filament. We have some evidence of increases in respiration when you add a very dense sperm suspension. In some cases there is a transient, but definite, increase in respiration (about double). Sperm with no eggs present don't give this.

TS'O: Anatomically, does the stimulation have to be in the head or tail of the sperm?

EPEL: Presumably, only the head can stimulate.

GROSS: The tail never hits first. There's apparently a strong chemo-taxis that orients the sperm in the direction of the egg so that the head goes first. This is important.

TS'O: Is this because of antibodies?

GROSS: Well, that's what Tyler says. There's a complicated literature. The assumption is that there is a specific receptor in the sperm, and that a product of the egg surface attracts the sperm toward the egg.

EPEL: There is good evidence for lytic enzymes in the acrosome which may be involved in getting into the egg. Whether these are involved in the activation isn't clear. In conclusion, the *in vivo* kinetic studies indicate that the timing of structural changes (light-scattering), acid excretion, electrical and fluorescence changes (TPNH) cannot at present be temporally separated from each other, but that these can all be temporally distinguished from respiratory activation. This, then, suggests both parallel and cascade-type reactions upon fertilization.

III. Universality of the temporal sequence

Because interspecies variations in behavior of other parameters after fertilization of sea urchin eggs have been found (18), it is important to determine whether the above changes occur in other species of sea urchin, and in the same sequence, or whether they are unique to the species so far described.

Figures 10 and 11 provide a partial answer to this question. The figures depict data, obtained in collaboration with Dr. Ray M. Iverson of the University of Miami, on the fertilization changes in the eggs of the sea urchin *Lytechinus variegatus*. Figure 10, which depicts respiration rate and acid excretion, shows the same temporal sequence in these two changes as had been observed in *S. purpuratus*. Of interest here is the rapidity of the acidity changes. In this species (at 30° C, as compared to 17° C for the

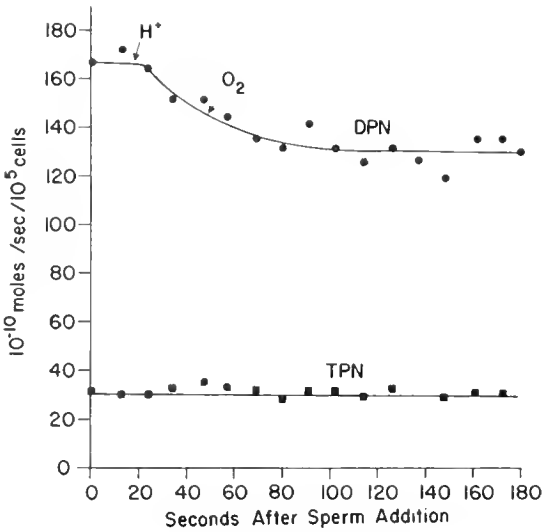

Fig. 11.

Analysis of DPN and TPN following fertilization of *L. variegatus*. Arrows indicate initiation of acid excretion and increased respiration (data of Epel and Iverson).

S. purpuratus), the acid excretion has begun at 18 seconds after sperm addition. The respiratory lag is longer here, O_2 consumption not increasing until 30 seconds after the pH increase.

Figure 11 shows that the DPN decrease similarly occurs, beginning after acid excretion and before respiratory activation. Although TPN does not change (analogous to the sea urchin *Arbacia punctulata*), TPNH does increase (data from separate experiments not shown here).

A similar temporal sequence was also observed in *Lytechinus pictus*, where measurements were done in the Pressman apparatus as in Fig. 8. It thus appears from an examination of two genera and three species, that the temporal sequence is identical as regards changes in structure, fertilization acid, fluorescence, and respiration.

IV. Significance and mechanism of observed changes

A. Light-scattering and acidity changes

The observed decrease in light-scattering suggested a volume or size increase. Although the volume of the egg supposedly does not change, there does occur an elevation of a "fertilization membrane". This membrane, in the unfertilized egg, lies closely apposed to a peripheral ring of granules - the cortical granules - which rupture upon fertilization, releasing their mucopolysaccharide contents. The overlying membrane is then presumably pushed out, or elevated, either by expansion of the mucopolysaccharide through hydration, through osmotic forces resulting from these substances, or molecular unfolding of the precursor membrane (see 19). At any rate, the effective volume of the egg doubles, which makes this change a prime suspect as the cause of the light-scattering change.

This hypothesis can be tested, since the precursor membrane can be removed with trypsin. When this was done - to our great surprise - the identical light-scattering change was still observed. The scattering change, therefore, does *not* result from elevation of the fertilization membrane. The two most plausible alternatives are that the scattering change represents either the breakdown of the cortical granules (which are trypsin-insensitive), or an actual change in cytoplasmic structure. The latter interpretation is suggested by changes in texture and granularity of the cytoplasm, which can be seen in stained eggs (20) or *in vivo* in extremely transparent eggs (21).

That the change might correspond to breakdown of the cortical granules is suggested by the similar kinetics of the acid excretion and light-scattering. Although we had initially thought the acid resulted from accumulation of some acidic carbohydrate compound (such as lactic acid), no compound analyzed was present in sufficient concentration to account for the acidity change. This was true for lactate, pyruvate, glucose-6-phosphate, 6-phosphogluconic acid, isocitrate, and malate. In fact, the only change so far described which can account for the acid production is the acidic mucopolysaccharide released by the cortical granules (22). If one assumes that the sulfate moiety of the mucopolysaccharide exists as sulfuric or bisulfuric acid in the granules, then the amount of protons released upon rupture of the granules would be in the same range as the observed acid release after fertilization (23). Although not yet proven, the similar stoichiometry and kinetics strongly support the conclusion that the light-scattering and acid increase result from the same event - the cortical granule breakdown.

Irrespective of interpretation, the kinetic analysis of the light-scattering changes suggests that structural changes may be highly critical in metabolic activation, since they are one of

the first observable changes. If they indeed do represent cortical granule breakdown, the hypotheses of Moser (24) and Runnstrom and Immers (25), relating granule breakdown to metabolic activation, take on added significance.

B. Respiratory changes

Although intensively studied since Warburg first observed the dramatic post-fertilization increase in O_2 consumption, the operative respiratory control mechanism is still unclear. One possibility, suggested by the work of Chance (26) and Lardy (27), showing respiratory control by phosphate acceptor (ADP), is that fertilization results in increased ATP utilization and concomitant ADP formation. The increased ADP level could then result in the increased respiratory rate. Such a hypothesis is also suggested by the recent finding that sea urchin mitochondria exhibit respiratory control via ADP (28). To check this possibility, eggs were sampled at rapid intervals after fertilization, and analyzed enzymatically for adenine nucleotides. The results of such assays, shown in Fig. 12, indicate no significant changes in these coenzymes. Most importantly, there are no changes at the time of maximum respiratory activation. Although this suggests that ADP-limited respiration (State 4-State 3 transition) is not operative here, it is probable that ADP produced is immediately rephosphorylated, and that perhaps it is the ADP content in the mitochondrial micro-environment which is critical.

An alternative possibility accounting for the low respiration rate in the unfertilized egg is that respiration is substrate-limited. If so, the increased respiratory rate following fertilization could result from increased availability of respiration-linked substrate [i.e., a State 2-State 3 transition, as defined by Chance and Williams (26)]. Such a mechanism was first suggested by the findings of Aketa *et al.* (29) that a large increase in the various glycolytic esters, especially glucose-6-PO_4, had occurred by five minutes after fertilization.

To check this possibility simultaneous analyses of respiration and glucose-6-PO_4 were carried out. The results of these experiments, shown in Fig. 13, indicate that such an interpretation might be tenable. It is seen that in *L. variegatus* the glucose-6-PO_4 level does indeed increase, and begins before the activation of respiration. This increase is rapid and large. By six minutes (not shown) it is six

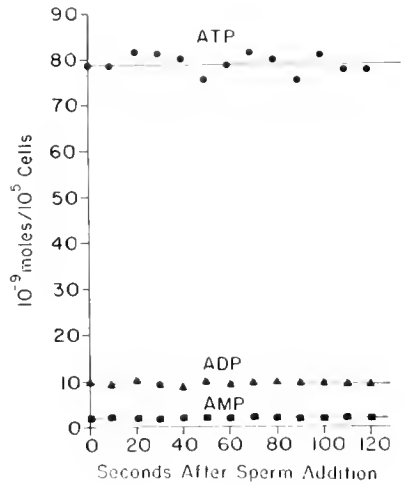

Fig. 12.

Adenine nucleotide levels following fertilization of *S. purpuratus*.

times the unfertilized level. Changes in glucose-6-PO_4 are nowhere near as marked in *S. purpuratus*, however, nor are they so obviously related to the respiratory activation. These differences could suggest that different substrates are being utilized in these two species, or that substrate mobilization is not critical to the respiratory activation. It could also mean that the different levels simply reflect differences in relative enzyme activities and rate of flux of the glycolytic substrates. For example, in frog skeletal muscle the glycolytic flux can increase many fold before any increase in glucose-6-PO_4 is seen (30), whereas in rat heart a flux increase is immediately reflected in a glucose-6-PO_4 increase (31). Since G-6-P is a substrate in flux, as opposed to a coenzyme which can cycle in its various forms, it might therefore be premature to ascribe too much importance to the different glucose-6-phosphate levels. Rather, the comparative results suggest that fertilization does activate substrate mobilization in both cases.

The enzyme(s) responsible for this mobilization is still not known. Glycogen phosphorylase is the best candidate, and is indeed present in both fertilized and unfertilized eggs of *S. purpuratus*. Furthermore, preliminary experiments indicate that the activity of this enzyme is sufficient to account for the peak respiratory activity of the fertilized egg.

POLLARD: Is all this respiration in the mitochondria?

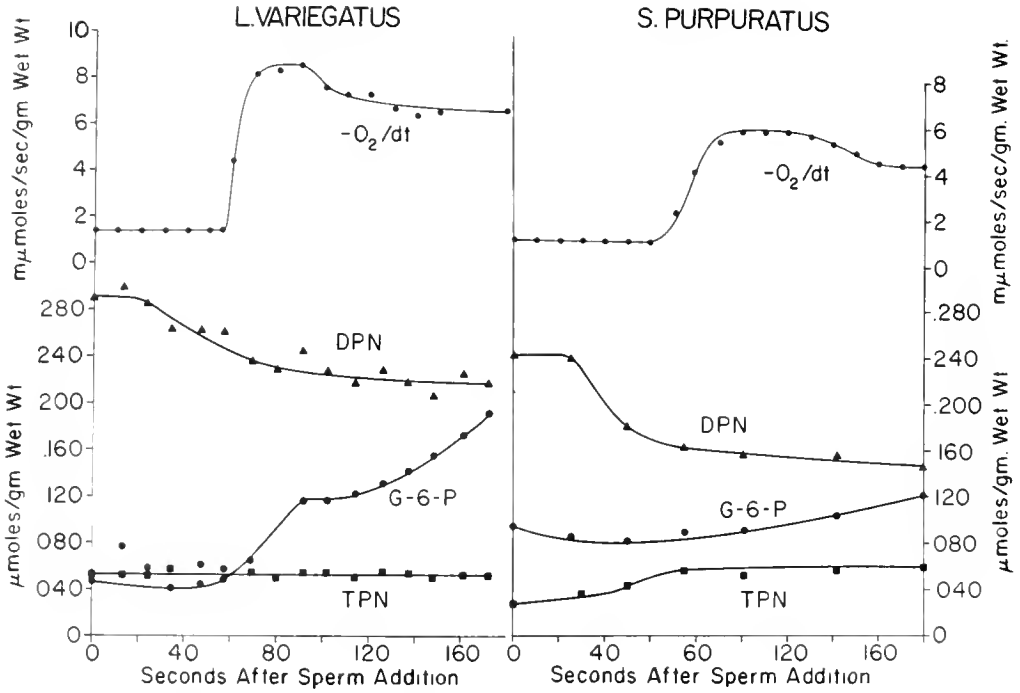

Fig. 13.

Comparison of changes in content of glucose-6-phosphate, DPN, TPN and rates of respiration in eggs of *L. variegatus* and *S. purpuratus*. (From Epel and Iverson, *In* "Control of Energy Metabolism," 1965; reproduced with permission of Academic Press.)

EPEL: We don't know yet, but it is another possibility. We're just looking into this now.

C. TPNH changes

The stoichiometry and kinetics of the pyridine nucleotide changes implicate activation of DPN kinase by fertilization. That this is the case is seen in Table III, which shows activity measurements of DPN kinase in homogenates prepared from unfertilized and fertilized eggs. As can be seen, the activity is essentially the same in both cases. Although this demonstrates that the enzyme is indeed present in the unfertilized egg, and hence activated by fertilization, it is disappointing from a heuristic viewpoint that these differences could not also be reflected in the broken cell preparations. This suggests that the enzyme is either activated by the homogenization procedures, that the enzyme is activated by the assay procedure, or that some substrate, activator, or cofactor missing in the unfertilized egg is being either released during homogenization or supplied in the assay mixture.

The structural changes, as well as several reports of enzyme translocation following fertilization (32, 33), suggested that the enzyme might be changing its subcellular site upon fertilization. To check this, the enzyme has been extracted with numerous different media, and the activity in particulate and soluble phases checked. In all cases, the enzyme has always been found in the supernatant.

Measurement of substrate localization before and after fertilization were also carried out, estimating the amounts of DPN and ATP in the mitochondrial-nuclear fraction and the post-mitochondrial supernatant. Although not completely satisfying from the viewpoint of both leakage of substrates from particles, and some loss of ATP and DPN during centrifugation, the results did not indicate any large amount of binding of ATP or DPN to or in particles. Briefly, 75% of the DPN and greater than 90% of the ATP were in the post-mitochondrial supernatant. As the DPN kinase is also in the supernatant, it appears that both substrates and enzyme are present in adequate amounts for the reaction to proceed. These findings, therefore,

TABLE iii

DPN Kinase Activity in Homogenates of *S. purpuratus*

Unfertilized	3.1 ± 0.1
Fertilized	3.2 ± 0.04

Eggs homogenized in 0.1 *M* triethanolamine buffer. 01 ml of this extract (1.1 to 1.3 mgms protein) was incubated for 30 minutes at 30°C in a medium containing 5 μmoles ATP, 5 μmoles DPN, 20 μmoles $MgCl_2$ and 180 μmoles triethanolamine buffer, pH 7.4, in a total volume of 2.0 ml. Assay procedures as in Fig. 14.

suggest that enzyme and substrate are "apparently available" to each other, but do not interact until after fertilization.

The remaining requirement for DPN kinase enzyme activity is a divalent cation. Although we as yet have no data on cation content of the soluble phase, Mazia (34) has shown that fertilization results in an increase in free Ca^{+2} (as opposed to bound, or non-dialyzable Ca^{+2}). Could the enzyme require Ca^{+2}, and if so, could the Ca^{+2} change account for enzyme activation?

Studies to test this hypothesis have been done by assaying enzyme activity in dialyzed or chromatographically desalted supernatants at ATP and DPN concentrations paralleling the *in vivo* concentrations of substrate. One such activity curve is shown in Fig. 14. It is seen that the enzyme exhibits a requirement for a divalent cation, and is activated more strongly by Ca^{+2} at low cation concentrations. Above 3mM, however, it is seen that Mg^{+2} activates 20% better than Ca^{+2}. Such behavior is relatively unique for a kinase, since most enzymes of this type are better activated by Mg^{+2}, and in some cases are Ca^{+2} inhibited. For example, Ca^{+2} is only 40% as active as Mg^{+2} in pigeon liver DPN kinase (11).

What picture emerges from these studies? The kinetic analysis suggests the following picture. A light-scattering change occurs, probably reflecting the breakdown of cortical granules. Coincident with this is the initiation of fertilization acid excretion, probably reflecting the release of sulfated mucopolysaccharides. Within a second or two of these two changes DPN kinase is activated. Shortly thereafter (or simultaneously) carbohydrate flux increases, possibly through phosphorylase activation, and when sufficient substrate has reached the respiratory chain, respiratory activation occurs.

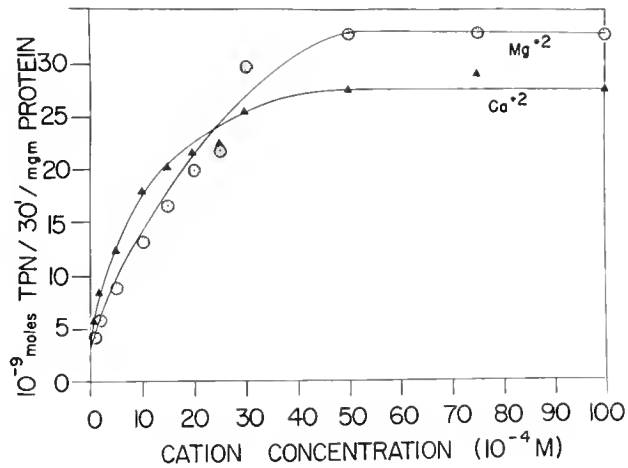

Fig. 14.

Cation dependence of DPN kinase from unfertilized eggs of *S. purpuratus*. 0.1 ml of a 12,500g supernatant, desalted by passage through a Bio-Gel P-2 column, was incubated with 0.2 mM DPN, 3.6 mM ATP and the noted concentration of cation in triethanolamine buffer, pH 7.4, 0.083 *M* for 30 minutes at 30°C. The reaction was quenched by boiling, and TPN assayed with isocitric dehydrogenase.

The above hypothetical scheme is compatible with the data. The weakest point is the picture of respiratory activation, since there is not really good evidence that carbohydrate mobilization via phosphorylase is the responsible factor.

Although the mechanisms for these changes are not rigorously defined, the analysis of the DPN kinase reaction suggests the hypothesis that the change in free Ca^{+2} is the primary activator of this enzyme. Glycogen phosphorylase can also be Ca^{+2} activated through the complex phosphorylase kinase system (35) so such a hypothesis takes on added interest. A possible criticism of this interesting theory is that the kinase is also Mg^{+2} activated, and the Mg^{+2} content *in vivo* is more than adequate to activate the enzyme (36). Although no data is available on the free Mg^{+2} content, it should be noted that the amount of RNA in the eggs is sufficient to completely bind the available Mg^{+2} (37). Obviously much more data has to be obtained in order to prove or negate the Ca^{+2}-activation hypothesis, and it is presented here solely to indicate the possible directions of this research.

In closing, I should like to comment on how the various post-fertilization reactions might be involved in initiating the syntheses characteristic of development. Monroy *et al.* (3) have recently reported evidence indicating

that activation of protein synthesis might be controlled through the transient proteolytic activity at fertilization. Here, the ribosomes are visualized as being coated by a protein envelope, thus preventing protein synthesis sterically. They visualize this envelope as being removed by the protease, thus resulting in increased protein synthesis.

The TPNH change could account for the observed activation of lipid synthesis at fertilization, since this coenzyme is specifically involved in this synthetic sequence. The TPNH change, especially the increase in the redox couple of TPNH/TPN, could also be critical for protein disulphide interactions believed to be involved in cell division (38). The total increase in the triphosphopyridine nucleotides could also be involved in the channeling of carbohydrate through the pentose shunt, whose activity increases following fertilization (39, 40, 9). A change in carbohydrate flux, although still not rigorously proven, could also be important in regulating macromolecule synthesis. Besides the important energy yields from carbohydrate metabolism, a major limiting factor could be carbon skeletons for synthesis, as, e.g., ribose for RNA synthesis.

V. Conclusions

The present study of the temporal sequence and mechanism of the fertilization reactions in sea urchin eggs has centered on light-scattering (structural) changes, fertilization acid excretion, and activation of DPN kinase and respiration. The data indicate that changes in light-scattering and acid excretion begin simultaneously, followed almost immediately by activation of DPN kinase. Respiratory activity increases last.

Analysis of these changes suggests that the light-scattering and acid changes reflect the breakdown of the cortical granules. DPN kinase activation might be through the free Ca^{+2} release known to occur after fertilization since this enzyme is both Ca^{+2} and Mg^{+2} activated. The mechanism of respiratory activation is still unclear, but the available data suggest substrate mobilization, possibly through control of glycogen phosphorylase.

POLLARD: Is there any possibility of getting at this genetically? Are there any deficient eggs which require that a large amount of calcium be added to the medium in order to get fertilization? This sort of thing would be something you could look at. There might be something here similar to the findings by Slonimski on yeast mitochondria, which themselves are rather specific kinetic things. Is this possible? Are sea urchins accessible genetically?

EPEL: Yes, generally they are. I think it would be a very good contribution. There may be some organisms in which you could do this. You do require calcium to fertilize invertebrate eggs in the sea water.

POLLARD: I feel you're trying to describe a lot of exciting kinetics without quite putting your finger on the initiating point.

EPEL: That's right.

POLLARD: You think that the best lead so far might very well be the potentiation of enzyme by action of calcium or magnesium, presumably initiated by some membrane component that makes this possible. You're referring, essentially, to a fast physical change, like chemiosmosis, followed by fairly rapid concentration of an ion which is favorable to enzyme X. I would feel that if you're starting to look at a single enzyme, this is the sort of thing that you could have missing genetically. Then you'd have to add a whole lot of other things to the medium to make it go. Is there any evidence at all for this sort of thing?

EPEL: Not that I know of.

TS'O: Is this enzyme stimulated by pH changes? For instance, will a simple change of pH from 6.9 to 6.5 affect the enzyme activity?

EPEL: No, it appears to have a broad optimum between pH 7 and 8.

TS'O: Can physical studies be made on fragments of membrane?

EPEL: There have been some enzyme studies made on sea urchin egg cell cortexes. They have a sodium-potassium-activated ATPase.

PAPACONSTANTINOU: This might implicate a regulation between the hexose monophosphate pathway and the Embden-Meyerhof pathway of glycolysis. We know that some substrates from the hexose-monophosphate pathway will regulate the activity of some glycolytic enzymes. I wonder whether there might be some regulation here where sedoheptulose-7-phosphate or other metabolites of this cycle affect the activity of this enzyme.

EPEL: Yes, I think this would be very possible.

ACKNOWLEDGEMENT

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RIBOSOMAL RIBONUCLEIC ACID SYNTHESIS IN *RANA PIPIENS* EMBRYOS

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One primary reason for the difficulty in studying the biochemistry of development is the lack of good genetic information on the developing systems which are normally used. It is now possible through the study of ribonucleic acid (RNA) synthesis to investigate the direct expression of a specific class of genes, ribosomal RNA (R-RNA) genes, during development. By utilizing developing *Rana pipiens* embryos we have attempted to get an insight into the gross aspects of the regulatory processes which control the synthesis of R-RNA during embryogenesis.

There were two technical problems to be solved before *Rana pipiens* could be used for the experimental animal in this study: 1) The utilization of standard ribosome isolation procedures resulted in the ribosomes being irreversibly bound to the egg proteins. It was found that the egg ribosomes could be readily isolated if the frog eggs were homogenized in a buffer of high ionic strength and high pH, to which sodium lauryl sulphate had been added (1). 2) When used on *Rana pipiens* eggs the usual methods for the isolation of undegraded high molecular weight R-RNA resulted in highly degraded low molecular weight R-RNA as the isolation product. It was obvious that large amounts of powerful nucleases existed in these eggs and a method had to be devised to negate the effect of these enzymes. This procedure primarily involved maintaining a temperature as low as possible during the RNA isolation procedure (1).

Three experimental embryological systems were used in this work to ask some simple questions about the regulative phenomena involved in the synthesis of ribosomal RNA during development. 1) Hybrid embryos were utilized in order to study the effect of a qualitative change in the genome of *Rana pipiens* on R-RNA synthesis during development. 2) Haploid embryos were employed to ascertain the effect on R-RNA synthesis during development of a quantitative change in the frog genome. 3) Embryos reared in a medium lacking in magnesium were studied to determine the effect of magnesium deprivation on R-RNA synthesis during development.

In order to have a base line for comparison of R-RNA synthesis in experimental systems to that in normal development, it was necessary to determine the pattern of R-RNA synthesis in the normally developing *Rana pipiens* embryo. Figure 1 depicts the pattern of R-RNA synthesis during normal development in *Rana pipiens*. R-RNA synthesis could not be detected during early development and was first detected at early gastrula stage (two left peaks in gradients shown). From early gastrula stage R-RNA synthesis increases rapidly as development proceeds. The base ratio of this newly synthesized RNA is high in guanine + cytosine which is a characteristic of all ribosomal RNA (Table I).

The first experimental system was picked in order to investigate the effect of a qualitative change in the *Rana pipiens* genome on the pattern of synthesis of R-RNA during development. Hybrid embryos produced by fertilizing *Rana pipiens* eggs with *Rana catesbeiana* sperm were used for these experiments. These hybrids developed normally until the onset of gastrulation and at this time development ceased. Although development ceased at the early gastrula stage, the hybrid embryos continued to live for several days (2). It was of interest to determine the pattern of R-RNA synthesis in the hybrid

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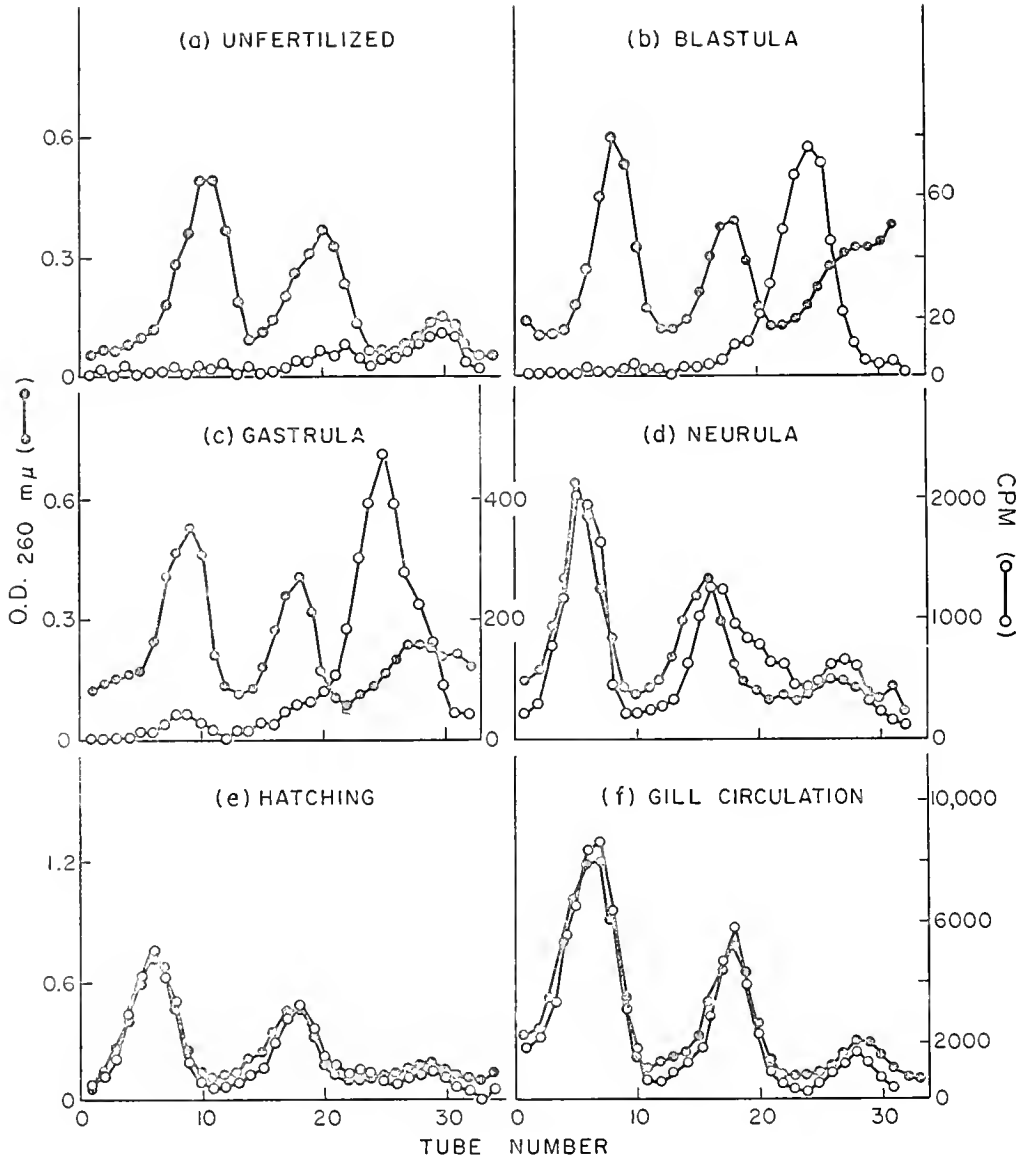

Flg. 1.

Sedimentation patterns of R-RNA extracted from ribosomes isolated from 200 ^{32}P-labeled: a) unfertilized eggs, b) blastula embryos, c) gastrula embryos, d) neurula embryos, e) hatching embryos, f) gill circulation embryos. Sibling embryos were used in this experiment.

embryos in the hope that it might yield some clue as to the control of R-RNA synthesis.

Twenty-four hour (early gastrula) and forty-eight hour (early neurula) ^{32}P-labeled control and hybrid embryos were extracted for RNA and the purified RNA preparation displayed on a sucrose gradient (Fig. 2). All RNA preparations were treated with DNase prior to sucrose density gradient analysis. It is evident from Fig. 2 that the hybrid embryos synthesize much less R-RNA at 48 hours than do the control embryos. There is some question as to whether the hybrid embryos synthesize R-RNA at all. Stained histological sections of hybrid and control forth-eight hour embryos showed nucleoli present in the control embryos but nucleoli were not observed in the hybrids. The sucrose density patterns, however, indicated that some R-RNA was synthesized in the hybrid embryos. Further work is necessary to resolve this point.

TABLE I

Base Compositions of Ribosomal RNA. a. Base composition of 28S and 18S R-RNA subunits. The values are expressed as mole per cent of the total RNA. b. The ^{32}P base composition of the ^{32}P-labeled 28S RNA isolated from early neurula embryos. Values are expressed as the per cent of the total CPM in the ^{32}P-labeled 28S RNA.

	Material		28S	18S
a.	Frog Eggs	2'(3') Uridylic Acid	19.5	22.3
		2'(3') Guanylic Acid	35.5	34.1
		2'(3') Cytidylic Acid	27.6	25.4
		2'(3') Adenylic Acid	17.4	18.2
	Adult	2'(3') Uridylic Acid	19.8	25.0
	Frog Liver	2'(3') Guanylic Acid	35.1	30.2
		2'(3') Cytidylic Acid	27.4	24.1
		2'(3') Adenylic Acid	17.4	20.8
b.	Early Neurula	2'(3') Uridylic Acid	17.3	
		2'(3') Guanylic Acid	34.3	
		2'(3') Cytidylic Acid	29.7	
		2'(3') Adenylic Acid	18.7	

In comparing R-RNA synthesis in *Rana pipiens* haploid embryos and the *Rana catesbeiana* x *Rana pipiens* hybrid embryos it is striking that the haploid embryos exhibit the normal pattern of R-RNA synthesis. The addition of a foreign set of chromosomes to the *Rana pipiens* haploid set of chromosomes has poisoned the hybrid embryo and rendered it incapable of further development. It is not likely that the crippling of the hybrid's ability to elaborate R-RNA was responsible for the developmental retardation and death of the embryo. Recent studies have shown that the anucleolate embryos of *Xenopus laevis* develop to the swimming tadpole stage in the complete absence of R-RNA synthesis (3).

The relative inability of the hybrids to elaborate R-RNA prompted us to utilize another experimental system. A developmental abnormality caused by rearing *Rana pipiens* embryos in medium lacking magnesium seemed to offer an approach to the problem of the control of R-RNA synthesis during development. Embryos reared in this manner (magnesium deficient embryos) develop normally to stage 21-23 (swimming tadpole) after which they undergo developmental retardation, become edematous and immobile and die 2-3 days later (4). Brown initially made several potentially interesting observations regarding the synthesis of ribosomes in these magnesium deficient embryos (4). The magnesium deficient embryos apparently contained one-sixth as much R-RNA in the *isolatable ribosome fraction* as did control embryos even though the magnesium deficient embryos contained the same amount of total RNA per embryo as did control embryos. Since R-RNA usually comprises 80-90% of the total cell RNA, it was of interest to investigate the nature of the RNA from the immobile magnesium deficient embryos.

Initial studies on the ribosomal content of magnesium deficient embryos demonstrated that an almost normal complement of ribosomes (as compared with control embryos) could be isolated if the ribosome extraction technique designed for *Rana pipiens* eggs was used. Further studies in which R-RNA was labeled while the

magnesium starved embryos were immobile indicated that immobile magnesium deficient embryos (Shumway stages 21-23, swimming tadpole) made fewer ribosomes than did control embryos of a comparable age (Fig. 3).

The apparent decrease in R-RNA synthesis in magnesium starved embryos could be explained by one or more of the following hypotheses: 1) Ribosomes were made at the normal rate in the magnesium starved embryos but ribosomal turnover was accelerated; 2) The rate of synthesis of R-RNA was slower in magnesium starved embryos than in controls; 3) There was a failure to assemble all newly made R-RNA into ribosomes.

The following experiment was performed to determine the stability of ribosomes in immobilized magnesium starved embryos. Embryos were grown in 10% Holtfreter's solution and at Shumway state 20-21 were labeled with $^{14}CO_2$ and then incubated for 20 hours in non-radioactive 10% Holtfreter's solution. At the end

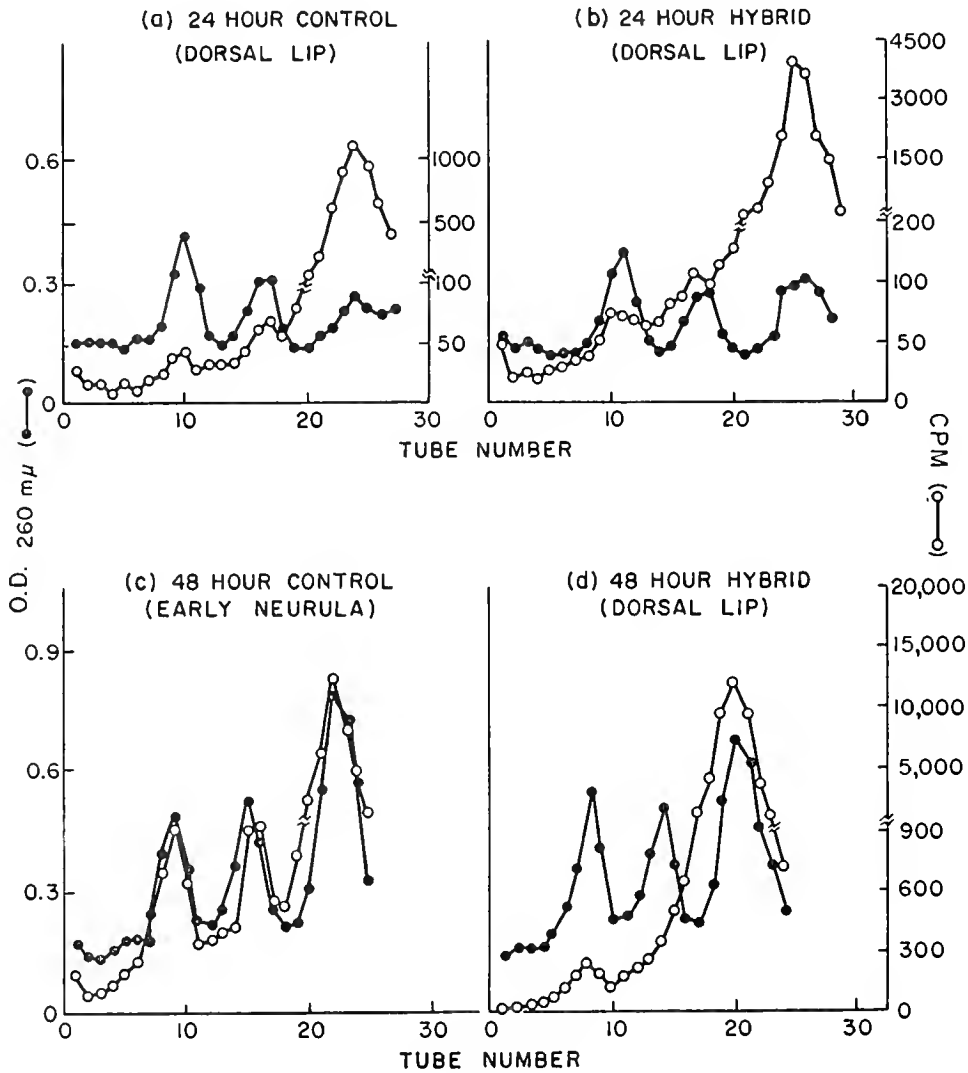

Fig. 2.

Sedimentation patterns of RNA extracted from ^{32}P-labeled whole control and hybrid embryos: a) 50 twenty-four hour control embryos, b) 50 twenty-four hour hybrid embryos, c) 55 forty-eight hour control embryos, d) 55 forty-eight hour hybrid embryos. Sibling embryos were used in this experiment.

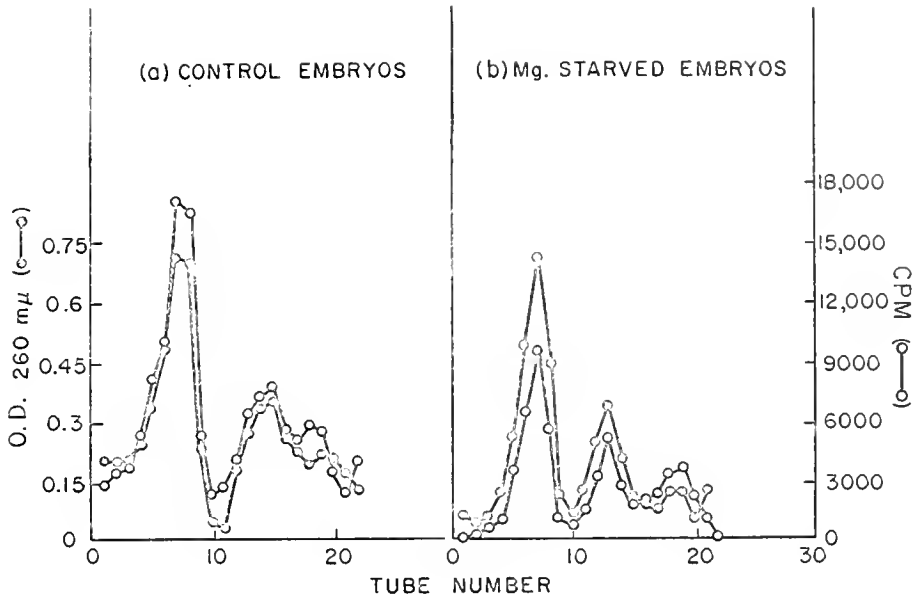

Fig. 3.

Sedimentation patterns of RNA extracted from ribosomes isolated from $^{14}CO_2$-labeled control and immobilized magnesium starved embryos: a) 25 control embryos, b) 25 magnesium starved embryos. The embryos were incubated for 1 hour in a solution containing 10 μc/ml of $Na_2{}^{14}CO_3$ and then placed in non-radioactive solution.

of the 20-hour "chase" the embryos were separated into three groups. RNA was extracted from the ribosomes isolated from one group (group 1) of embryos. A second group (group 2) was placed in 10% Holtfreter's solution containing magnesium. The third group (group 3) was placed in 10% Holtfreter's solution which lacked magnesium. The second and third groups of embryos were kept in their respective solutions for three days, at which time ribosomes were isolated from each group of embryos and extracted for RNA. The magnesium starved embryos were immobile by the end of three days. The RNA obtained from each group of embryos was analyzed by sucrose density gradient centrifugation. If the ribosomes of the magnesium deficient embryos were stable, the amount of radioactivity present in the R-RNA of immobilized magnesium starved embryos would be identical to the amount of radioactivity present in the R-RNA of an equal number of embryos from each control group (Group 1 and group 2).

The amount of radioactivity present in the R-RNA of immobile magnesium deficient embryos was equal to the amount of radioactivity present in the R-RNA of group 2 embryos and very nearly equal to the amount of radioactivity present in R-RNA of group 1 embryos (Figs. 4a, b, c). This demonstrated that the magnesium starvation syndrome did not affect the stability of normal ribosomes.

This same experiment also indicated that the synthesis of ribosomes was slower in magnesium starved embryos as compared to control embryos. The specific activities, measured in counts/minute/unit of optical density at 260 mμ (CPM/OD), of R-RNA from group 1, 2 and 3 embryos were presumably identical at the end of the 20-hour chase. Since no more radioactivity was available for R-RNA synthesis in group 2 and 3 embryos (the total radioactivity incorporated into the RNA was nearly the same for each group), any further synthesis of R-RNA would result in a dilution of the radioactivity and a reduction in the specific activity of the R-RNA. The specific activities reported here were calculated from the amounts of radioactivity and optical density present in the peak tube of the 28S R-RNA component of each of the three groups. The specific activity of the R-RNA of group 1 embryos was 16,700 CPM/OD (Fig. 4a). Group 2 R-RNA had a specific activity of 9700 CPM/OD (Fig. 4b), while the R-RNA from

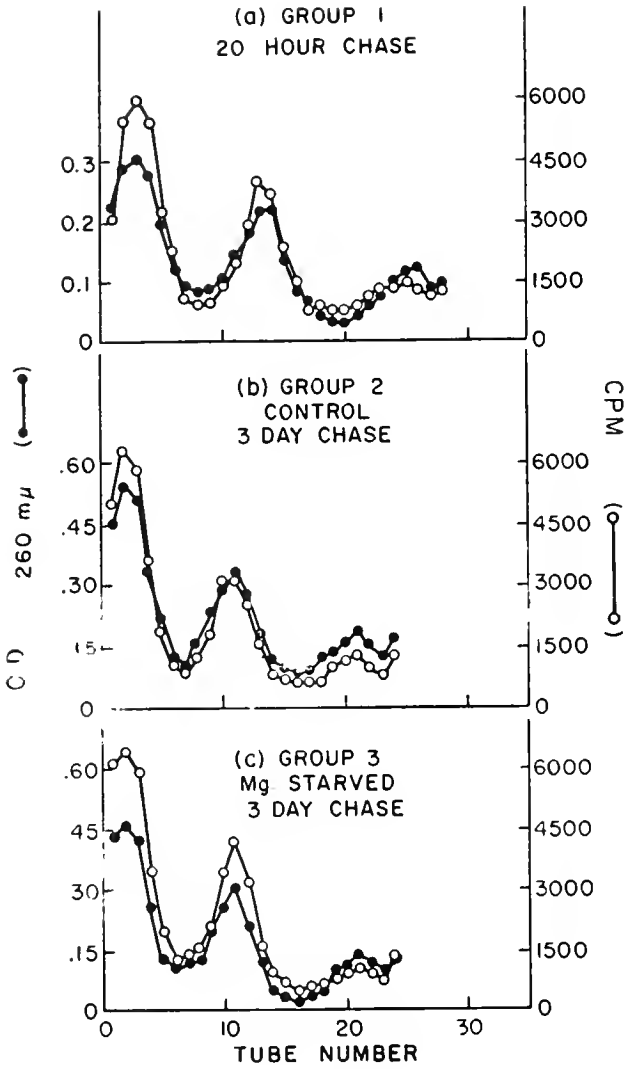

Fig. 4.

Sedimentation patterns of RNA isolated from $^{14}CO_2$-labeled control and immobilized magnesium starved embryos: a) 35 Group 1 control embryos, b) 35 Group 2 embryos, 3 day chase, c) 35 Group 3 magnesium deficient embryos. The embryos were incubated for 1 hour in a solution containing 10 μc/ml $Na_2{}^{14}CO_3$. Sibling embryos were used in this experiment.

magnesium deficient embryos had a specific activity of 11,700 CPM/OD (Fig. 4c). Since the specific activity of the R-RNA of the group 2 embryos was lower than specific activity of the R-RNA from magnesium deficient embryos the group 2 embryos were making more ribosomes than the magnesium starved embryos.

The question still remained whether the magnesium starved embryos converted all newly synthesized R-RNA into ribosomes. When actinomysin D was used to inhibit RNA synthesis in HeLa cells, the majority of the newly synthesized R-RNA remained in the nucleus in the form of 28S and 18S R-RNA subunits and was not assembled into ribosomes. Some of the R-RNA was assembled into ribosomes which were transferred to the cytoplasm (5). An experiment was performed to test the possibility that a similar situation existed in magnesium deficient embryos.

Control and immobilized magnesium deficient embryos were labeled with $^{14}CO_2$ for 1 hour and then placed in non-radioactive medium for a 20-hour "chase". RNA was extracted from control and magnesium starved embryos and ribosomes isolated from control and magnesium starved embryos. The RNA was then analyzed in a sucrose density gradient. The specific activities of the 28S R-RNA peaks were determined for each sample. If the ratio of the specific activities of whole egg 28S RNA/28S RNA extracted from isolated ribosomes was appreciably higher for magnesium starved embryos than the same ratio for control embryos, it would indicate that the magnesium starved embryos have difficulty in assembling newly made R-RNA into cytoplasmic ribosomes.

The value of the ratio was 0.98 for magnesium starved embryos and 0.97 for control embryos. These figures indicated that no more newly synthesized R-RNA was accumulated in the nuclei of magnesium starved embryos than was accumulated in the nuclei of control embryos.

The data presented here indicate that immobilized magnesium deficient embryos contain almost normal amounts of ribosomes and are capable of synthesizing ribosomes. These magnesium starved embryos, however, made fewer ribosomes than did control embryos of the same chronological age. The experiments on magnesium deficient embryos in this report were based on the assumption that ribosome synthesis in the magnesium deficient embryos was, somehow, impaired. It must be remembered, however, that a characteristic of the magnesium starvation syndrome is partial developmental arrest of the magnesium deficient embryos. Control and magnesium starved embryos of the same chronological age were not at the same developmental stage. It is possible that the magnesium deficient condition had no effect at all on the rate of synthesis of ribosomes and that the rate of ribosome synthesis observed in the magnesium deficient embryos was characteristic of all embryos at that developmental stage.

Rana pipiens haploid embryos were next investigated in our search for some clue to the mechanism of control of R-RNA synthesis during embryogenesis. These embryos were useful for studying the effects of a quantitative change in the *Rana pipiens* genome on R-RNA synthesis during development. Haploid embryos were produced by fertilizing normal *Rana pipiens* eggs with ultraviolet irradiated sperm. The subsequent haploid embryos exhibited all of the characteristics usually associated with the "haploid syndrome."

Rana pipiens haploid embryonic development is characteristically abnormal and delayed as compared to control embryos. Development proceeds normally until late blastula, at which time the haploid embryos begin to show developmental retardation. Haploids continue to develop for eight days at which time the majority of the embryos become edematous and die (6, 7). Cytological studies demonstrated that the normal sized cells of the control embryos contained a diploid set of chromosomes and two nucleoli. The smaller cells of the haploid embryos contain one nucleolus and a haploid set of chromosomes. These haploid cells, as expected contain one-half as much DNA as diploid cells (8).

It was possible to study the effect of quantitative changes in the gene complement of developing embryos on R-RNA synthesis by investigating the synthesis of R-RNA in haploid embryos. Four- and six-day old ^{32}P-labeled control and haploid embryos were analyzed for RNA, DNA and incorporation of ^{32}P into R-RNA. Developmental retardation, characteristic of haploidy, necessitated still another type of control. Haploid and normal embryos of the same chronological age were not the same developmental age since the haploids developed at a slower rate. The additional control consisted of five-day old normal embryos, which closely approximated the same developmental age as the six-day haploid embryos. Both haploid and control embryos originated from the same clutch of eggs. Tail tips of these embryos were also examined cytologically to determine the number of nucleoli per cell.

Quantitative determinations demonstrated that considerable RNA and DNA synthesis occurred in both haploid and control embryos between four and six days of development (Table II). The RNA increase was almost directly proportional to the DNA increase in both haploid and control embryos (Table II). Sucrose density gradient analysis also indicated that R-RNA was being synthesized in both haploids and controls

TABLE II

The values in this table arise from the experiment illustrated in Fig. 2. An aliquot was taken from the whole homogenate of each set of embryos and assayed for RNA and DNA. All values are given on a per embryo basis.

Stage	µg RNA	µg DNA	µg RNA / µg DNA
4-day Haploid	4.1	2.9	1.41
6-day Haploid	7.4	6.6	1.12
4-day Control	5.8	4.2	1.38
5-day Control	6.7	5.6	1.20
6-day Control	14.4	12.6	1.14

(Fig. 5). As expected, control embryos contained more RNA and DNA than did haploid embryos of the same chronological age (Table II). Haploid embryos (6-day) contained nearly the same amount of DNA and RNA as did control embryos (5-day) of about the same developmental age (Table II). Cytological examinations demonstrated the presence of one normal sized nucleolus per cell in haploid embryos while the larger cells of the control embryos contained two nucleoli.

The RNA/DNA ratios of both haploid and diploid embryos were approximately the same at all stages checked (Talbe II). This indicated that a unit of DNA produced about the same amount of R-RNA whether it resided in a haploid cell or a diploid cell. Since the cells of haploid embryos contained only one-half as much DNA as the cells of diploid embryos, the cells of haploid embryos produced only one-half as much R-RNA as the cells of diploid embryos.

Haploid embryos were developmentally retarded and it was expected that they would contain less RNA and DNA than control embryos of the same chronological age. It was, however, surprising that haploid embryos contained approximately the same amount of RNA as control embryos of the same developmental age. These results implied that the amount of RNA synthesized during development was a function of the stage of development. Brown reached a similar conclusion in studies on *Xenopus* haploid embryos where the haploid embryos also contained the same amount of RNA as control embryos of a comparable developmental age (9).

Haploid and diploid embryos of the same developmental age also contained about the same amount of DNA. Haploid embryos, then, had

roughly twice the number of cells as did diploid embryos at a comparable developmental stage. It has been shown elsewhere that triploid cells are 3/2 as large (10) and contain 3/2 as much DNA as diploid cells. This implies that triploid and diploid embryos of the same size contain the same amount of DNA, since triploid embryos contain two-thirds as many cells as diploid embryos and each triploid cell has 3/2 as much DNA as a diploid cell.

Indirect evidence suggests that the amount of DNA necessary to reach any developmental stage is a function of the volume of the egg from which the embryo originated. Frog embryos originating from small eggs, consisted of a reduced number of normal sized cells as compared to control embryos originating from normal sized eggs (12). The smaller embryos contained fewer cells and, therefore, probably less DNA than did the larger embryos at the same developmental stage. A reduction in the amount of cytoplasm per embryo thus produced a proportional decrease in the DNA content per embryo as compared to normal sized controls. These considerations suggest that during development the extent of DNA synthesis is regulated by the amount of cytoplasm present in the embryo and that this regulation is reflected by the similar DNA/cytoplasm ratios of haploid, diploid and triploid embryos.

Since haploid and diploid embryos of the same developmental stage contain about the same amount of DNA, it is possible that the stage of an embryo is dependent on the DNA content of that embryo. That is, a certain quantity of DNA (relative to the amount of cytoplasm present) must be present in an embryo before the embryo can attain a specific developmental stage.

We have seen that the DNA content seems to be controlled by the amount of cytoplasm present in the egg and that R-RNA synthesis is apparently stage dependent. With these observations in mind a hypothesis concerning the gross regulation of R-RNA synthesis during development follows. Specifically I would suggest that during development the extent of DNA synthesis is controlled by the amount of cytoplasm present in the embryo and that an interaction between the DNA and the cytoplasm somehow regulates the synthesis of R-RNA. It is now possible to design experiments to directly test this hypothesis.

POLLARD: Have you tried any microinjections? You could just mash up an ordinary embryo, one that won't arrest in two or three days, separate out the enzyme part and inject it into the mutant. This is based on the possibility that a "transcriptase" for making ribosomal RNA is missing.

KOHNE: Usually when you inject anything into these embryos, they arrest all by themselves. It's very difficult to put anything into an egg because you get chromosomal abnormalities.

POLLARD: If these are already arrested, you've got nothing to lose.

KOHNE: There *is* something that more or less approximates what you're asking. I haven't the vaguest idea what it means but Briggs at Indiana has an axolotol mutant that he calls the "00" or something similar. This mutant even looks different during early development, but it will develop into a gastrula and then become arrested. However, if you take normal egg cytoplasm and inject it into this mutant, it develops beautifully.

POLLARD: Maybe that "loosens up" the transcription.

GROSS: With this technique you get a gastrula arrest and a failure of the ribosomal RNA synthesis to turn on? However, you're not suggesting that it's the failure of ribosomal RNA synthesis to turn on that is responsible for the gastrula arrest, are you?

KOHNE: No, I think the evidence from the anucleolate mutant says that ribosomal RNA is not needed yet, at least until stage 21.

PAPACONSTANTINOU: Are you familiar with the experiments that Stanley Cohen did a few years ago in regard to this arrest? He looked at the respiratory cycle intermediates and found an accumulation of malonic acid in these embryos. I don't know if anybody has repeated them. but I know they are in the literature. You may have a lesion in the respiratory function and, if this is the case, you may be able to repeat this with your controls by adding malonate.

KOHNE: There's one other comment on this that I'd like to make with respect to hybrids of the *Rana catesbeiana* sperm x *Rana pipiens* egg cross, which have a haploid set of *Rana* chromosomes but arrest at gastrula. Haploids which have the *Rana pipiens* chromosomes develop almost normally until the swimming tadpoles. Thus, the *catesbeiana* chromosomes are doing something that is poisoning the system.

PAPACONSTANTINOU: Does it always have to be the *catesbeiana* male and the *pipiens* female? Can it be the other way around?

KOHNE: Yes, but they arrest, too. There are a lot of hybrid embryos it would be interesting to work with but the problem is getting the material. *Rana sylvatica* is one where you

get a hybrid arrest at gastrula. Nucleoli then start forming and the embryo lives for several days but remains at the gastrula stage. This system would appear to be well suited for this type of study. We could not, however, obtain any of the frogs.

PAPACONSTANTINOU: Another question relates to the volume in these embryos as they approach gastrula. Is the total volume the same?

KOHNE: Well, it may not be but if there is a change it's so imperceptible that you don't notice it.

PAPACONSTANTINOU: If you looked at this with an electron microscope, you wouldn't be able to detect a decrease in the ribosomal population per cell as it develops from the egg to the gastrula? You're not synthesizing ribosomes, but your cells are dividing. I don't know how many cells there are in a gastrula state but I was wondering whether the total volume of the whole embryo remains the same.

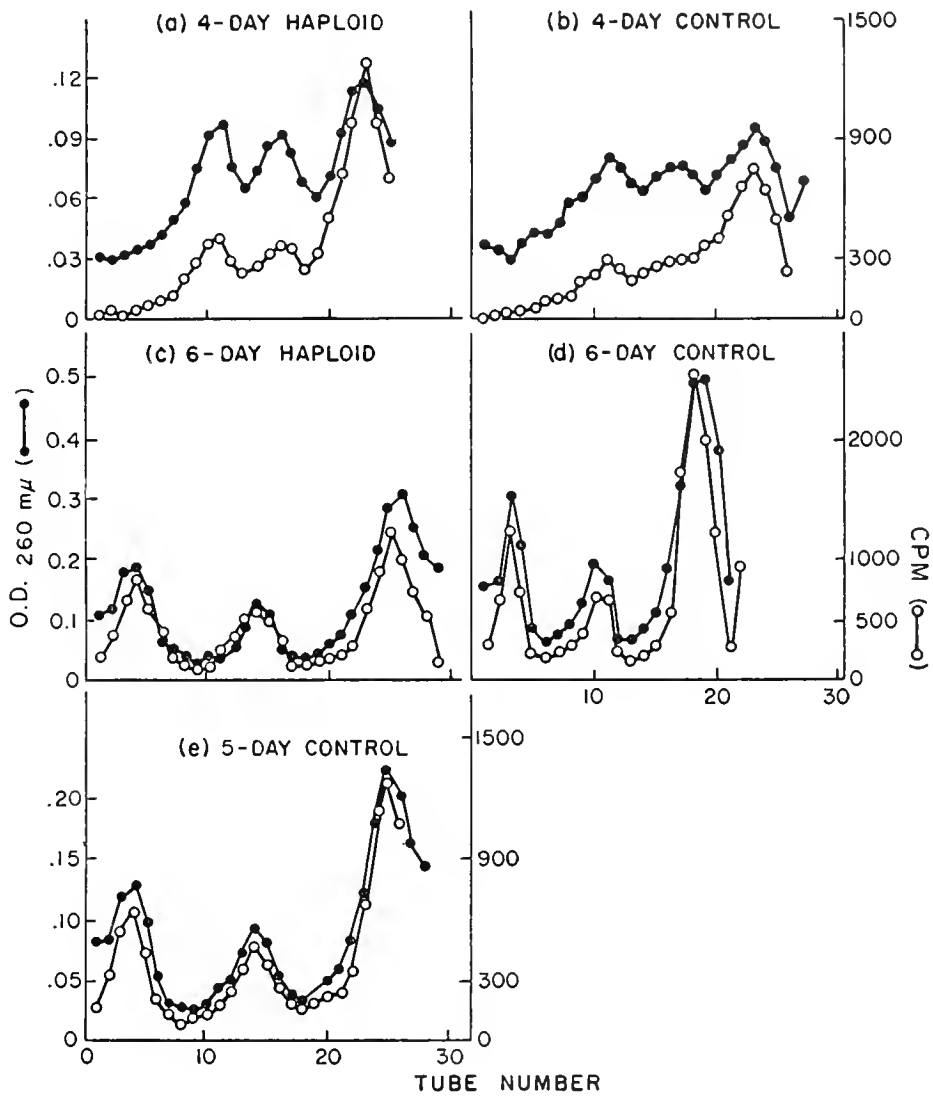

Fig. 5.

Sedimentation patterns of RNA extracted from whole ^{32}P-labeled haploid and control embryos: a) 20 four-day-old haploid embryos, b) 20 four-day-old control embryos, c) 20 six-day-old haploid embryos, d) 20 six-day-old control embryos, e) 20 five-day-old control embryos. Sibling embryos were used in this experiment.

KOHNE: In histological studies, from what I've seen, the cells look about the same size.

KAHN: Does developmental arrest of the *catesbeiana-pipiens* hybrids occur at the same stage in reciprocal crosses?

KOHNE: No, it's different. An interesting thing about hybrids in general is that they react differently depending on which egg cytoplasm is used. Even though the same total genome is present in the different cytoplasms the end result may be quite different.

EPEL: Are the magnesium-deficient and the anucleolate embryos the same?

KOHNE: No, but they are almost phenocopies. They act more or less the same way, except the magnesium-deficients obviously do synthesize ribosomal RNA.

POLLARD: Do you have any kind of hypothesis? For example, can we say the following? The idea is that one chromosome or one part of a chromosome has the mechanism for transcribing the ribosomal RNA. This has to come off the DNA. The DNA you have is no good, it won't work. It'll transcribe all right in one, but it won't in the other, so it's stuck with the wrong transcription. It keeps pumping this out and this hooks up with the RNA and it doesn't work. The stage in which you need this ribosomal RNA could come quite a bit earlier than when the cell is desperate for it.

KOHNE: Yes, I agree with you.

POLLARD: The concentrations may be very critical. You may need just 8 or 10 ribosomes to get something started. It seems to me you've got to have the organism *tell you* when you're supplying a deficiency. I would start with a nice arrested cell with everything in bad shape and then start firing things into it. I would use anything I could think of that was remotely similar to transcriptase, anything I could get off a DNA, and any kind of histone material which was somehow associated with DNA. I'd try to get hold of something that would unlock this mechanism. What's the matter with that idea?

KOHNE: Well, I'm overwhelmed.

GROSS: Dr. Pollard, why do you fire in transcriptase?

POLLARD: Well, it seems to have everything else.

GROSS: It's also got transcriptase.

POLLARD: Yes, but what it has won't work.

GROSS: I think I'd try naked ribosomes.

POLLARD: Would you use ribosomes made on the DNA in the hybrid?

KOHNE: The haploid is perfectly capable of making ribosomes; the only thing the haploid doesn't have that this hybrid has is the other set of chromosomes.

POLLARD: Maybe you've got the right transcriptase and the wrong DNA.

KOHNE: Well, each of these things contributes a nucleolus. I think that the cytoplasm interaction with this *catesbeiana* genome is what stops development. However, what it is really doing I don't know.

PAPACONSTANTINOU: If the nucleoli don't appear when they should appear, right before gastrulation, I don't understand why you think it has to be cytoplasmic. Do you think something in the cytoplasm is regulating the appearance of the nucleolus?

KOHNE: Yes, let me talk about that in a minute. This is all really pretty much speculation. We don't have many facts to go on.

TS'O: Have any practical chemical tests been done here?

KOHNE: I don't think anybody's ever done any on *catesbeiana*. That's something I've always wondered about. What differences are there qualitatively and quantitatively in the DNA's of these frogs. Amphibians have a lot of DNA in their cells compared to other things.

GROSS: Let us go back to some of your other points. Is there a fixed quantitative relationship between cytoplasm, DNA and RNA? Maybe there is a cytoplasmic repressor.

KOHNE: Possibly. I'm just saying that cytoplasm is somehow involved in control of nucleic acid synthesis. These cells also divide extremely rapidly during development.

POLLARD: You're saying that up to tetraploid in your system, the relationship of cytoplasmic volume to RNA and DNA seems to be very constant?

KOHNE: I was quoting other people's work. In 1925 de Beer quoted experiments related to cytoplasmic-nuclear ratio. He suggested that what controls the development of embryos is the cytoplasmic-nuclear ratio. He stated that when you get to a certain stage the genome is turned on and that what triggers it is the ratio of the nuclear material to the cytoplasmic material. He pinpointed that stage at gastrula, the same stage at which we know now that messenger RNA synthesis is turned on very rapidly. I have changed the wording a little to the parlance of molecular biology in saying that the cytoplasm is controlling the extent of DNA synthesis and that a DNA-cytoplasm interaction controls RNA synthesis.

DEERING: Does this 6-day haploid have twice as many cells as the 5-day control?

KOHNE: That's right.

DEERING: Are the cells smaller?

KOHNE: Yes, they are. Again this is a generalization because I can't say anything about what happens to the cells of specific parts, such as those destined to be the liver, brain or epidermis.

GRUN: There is one thing I was wondering about. This is the comparison between haploids and diploids. I wonder, has any attempt been made to produce inbred lines of frogs? What I'm concerned with is the question of whether this is a straight line comparison of the haploid state as compared with the diploid state or whether you're saying that the condition is a genetic effect of exposed recessives that, of course, would be effective in the haploid state and cause abnormal development.

KOHNE: Well, as I mentioned, if you start with a small enough egg, you will get normal development, so the phenomenon is probably not caused by the expression of recessives.

KAHN: Have you considered the possible role of cytoplasmic DNA?

KOHNE: There hasn't been any evidence as yet that cytoplasmic DNA is in any way active. Igor Dawid at Carnegie Institution has isolated a substance that has the characteristics of non-nuclear DNA, and he thinks it may come from mitochondria. Whether it's active in the differentiation process I don't know.

KAHN: Isn't it true that DNA synthesis does not begin until the beginning of gastrulation?

KOHNE: No, it begins immediately. Many people have thought that the cytoplasmic DNA might be contributing to the genome, but it's never been proven. There are several systems now in which immediate DNA synthesis has been shown.

GROSS: It doesn't make any difference quantitatively - the new DNA in the amphibian doesn't begin to make an impact on the total DNA per egg for some time.

KOHNE: There is supposed to be about 1000 times more cytoplasmic DNA than nuclear DNA in *Rana* eggs and 300 times in sea urchin.

GROSS: At any rate, there is a lot of it around, and even if each genome is fully replicated from the pool, the impact on the total DNA will be small until you get about a thousand cells or so. His ratios are all taken at stages where, presumably, the cytoplasmic DNA has been used up.

KAHN: This raises other questions. How is the cytoplasmic DNA utilized? What is the function of mitochondrial DNA?

KOHNE: There are two sources of diphenylamine-reacting material (DNA like). One of them is the mitochondrial DNA. The other one is an acid soluble fraction and is probably just nucleotides. There is about 10 times as much of the latter as there is of the DNA polymer.

GROSS: It may turn out that the thing people have been overlooking systematically is the enormous ratio of cytoplasm to nucleus in the egg. Since the sizes of mitochondria don't differ greatly between embryonic and somatic cells, this may mean that in the egg you have thousands of times as many mitochondria per nucleus as you have in the somatic cell. And if all mitochondria do have DNA, then in the egg the mitochondrial DNA might make a tremendous impact on the total, whereas in a somatic cell it wouldn't.

KOHNE: There is some initial circumstantial evidence, in studies on centrifuged *ascidian* eggs, that you get two fractions: one of them with mitochondria and the other without. The part with mitochondria will develop and the part without won't. For another type of *ascidian* with a light mitochondrial fraction and a heavy mitochondiral fraction, you get a partitioning of mitochondria in each fraction and both will develop: one of them being haploid and the other being diploid. However, without the mitochondria these things don't develop.

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MOLECULAR ASPECTS OF LENS CELL DIFFERENTIATION

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I. Introduction

We spent the first session of this workshop discussing some of the molecular aspects of early embryonic differentiation. Through these discussions it has become obvious that one of the major problems confronting the investigators studying the mechanisms of cellular differentiation is how developing cells acquire specific biochemical characteristics and how these are linked to morphological development and cellular function. It is now well documented that as cells progress through specific stages of differentiation new biochemical traits can be acquired and some existing traits can be lost. Thus, during differentiation there occurs a progressive cellular diversification which is characterized morphologically by cellular structure and biochemically by the synthesis of specific structural proteins and enzymes. The ultimate form of morphological and biochemical specialization may be seen in the muscle cell, erythrocyte, lens cell, etc., which synthesize tissue specific proteins in the form of myosin, hemoglobin and crystallins, respectively. This ability of cells to lose and acquire specific biochemical characteristics during differentiation is attributed to differential gene action. The mechanisms by which vertebrate cells can regulate genetic expression are not known; however, it is these mechanisms which are believed to be fundamental to the regulation of morphogenesis. One of the approaches to the study of these mechanisms is through studies on the regulation of synthesis of tissue specific proteins as cells become more highly differentiated. This afternoon I would like to start the session by describing a system in which the regulation of synthesis of specific proteins is associated with a specific stage of cellular differentiation, i.e., the differentiation of the lens epithelial cell to the fiber cell. In addition, I would like to describe a series of changes in the nucleic acids (RNA and DNA), also associated with fiber cell formation and possibly associated with the regulation of protein synthesis. Our studies have been centered, therefore, on the occurrence of protein and nucleic acid changes associated with a specific stage of lens cell differentiation. Before proceeding to discuss our biochemical data I would like to go over the morphological changes which occur in these cells and then associate these changes with the biochemical events.

II. Morphological Changes in Fiber Cell Differentiation

A. Structure of the lens

The lens is an avascular tissue composed of the following distinct cell types: (a) an outer single layer of epithelial cells; (b) a zone of elongation, composed of cells which are in the process of developing into fiber cells; and (c) the inner fiber cells (Fig. 1). Initiation of the differentiation of epithelial cells to fiber cells occurs at the peripheral or equatorial zone of the lens. It is in this region where the gross morphological changes associated with fiber cell differentiation occur, i.e., the transition from a cuboidal lens epithelial cell to the elongated fiber cell. After the embryonic lens has been formed, fiber cells are continuously laid down throughout the pre-natal and post-natal life of the animal. The bulk of the lens is composed of layer upon layer of these fiber cells, and this continuous formation of fiber cells accounts for the growth of this tissue. It can be seen, therefore, that (a) secondary

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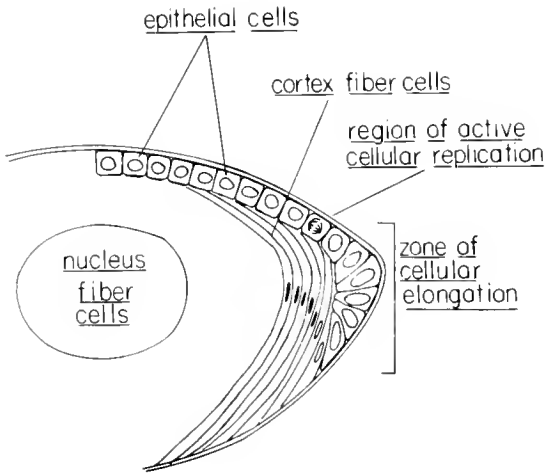

Fig. 1.

A diagramatic presentation of the adult vertebrate lens. The lens is surrounded by an external non-cellular capsule. Beneath the capsule are found the lens epithelial cells. The zone of cellular elongation is found in the peripheral area. This is the region of transition where the epithelial cells begin to elongate into fiber cells. The fiber cells that are newly laid down represent the cortex region; the fiber cells laid down during the early growth period of the lens compose the nucleus region of the adult lens. (Fig. 1, J. Papaconstantinou, *Science*, In press; Copyright 1966 by the American Association for the Advancement of Science.)

fiber cell formation represents the final stage of lens cell differentiation and (b) in the adult lens the fiber cells formed during embryonic growth compose the central or nucleus region while the newly formed fiber cells are found in the peripheral or cortex region.

B. Cytological and cytochemical observations on the process of fiber cell formation

The lens epithelial cells are characterized by their cuboidal shape, their basophilic staining properties and their ability to replicate (1). In the zone of elongation (Fig. 2), where the epithelial cells begin the process of fiber cell formation the following changes occur in the intracellular structures: (a) the cell sends out cytoplasmic processes anteriorly and posteriorly beneath the cuboidal epithelial cell layer to form the fiber cell; (b) the nucleus and nucleoli enlarge (2); (c) the ribosomal population increases significantly, especially in the cytoplasm adjacent to the enlarged nucleus (3, 4).

In the completed fiber cell, (a) the cytoplasm loses its basophilic properties and takes on acidophilic properties; (b) the nucleus and nucleoli reduce in size and the endoplasmic reticulum, which has a granular appearance in the epithelial cell, takes on a smoother appearance in the fiber cell; (c) through electron microscope studies it has been shown that a significant decrease in the ribosomal population occurs in the differentiated fiber cell (3, 4). These differences in staining properties and changes in intracellular structures indicate that significant macromolecular changes are associated with fiber cell differentiation. The enlargement of the nucleus and nucleoli, for example, as well as the increase in ribosomal population are an indication of increased nucleic acid and protein synthesis during elongation. Keeping these structural changes in mind, I would like to describe a series of biochemical events which are associated with fiber cell formation, and which may be closely linked with the cytological observations just described.

III. The Biochemistry of Lens Fiber Cell Differentiation

A. The association of γ-crystallin synthesis with fiber cell differentiation: gene activation

I would like to begin this section of my discussion by describing our observations on the appearance of a group of lens proteins, the γ-crystallins, during the differentiation of the lens epithelial cell to a fiber cell (5, 6). This presents us with an example of the activation of the synthesis of a specific protein simultaneously with the initiation of the morphological changes associated with the differentiation of a fiber cell. There are three major groups of proteins synthesized by lens cells; the α-crystallins, β-crystallins and γ-crystallins. The crystallins were first classified according to their mobility at alkaline pH; the fastest migrating group being the α-crystallins, the intermediate group being the β-crystallins and the slowest migrating group being the γ-crystallins (7, 8). More recently, through the efforts of my colleagues and myself, these structural proteins have been identified according to their elution properties on DEAE-cellulose columns (5, 9). It was essentially through the resolving power of DEAE-cellulose that the qualitative and quantitative differences in the crystallins of the different lens cells were detected. Typical patterns showing the stepwise elution of α-, β-

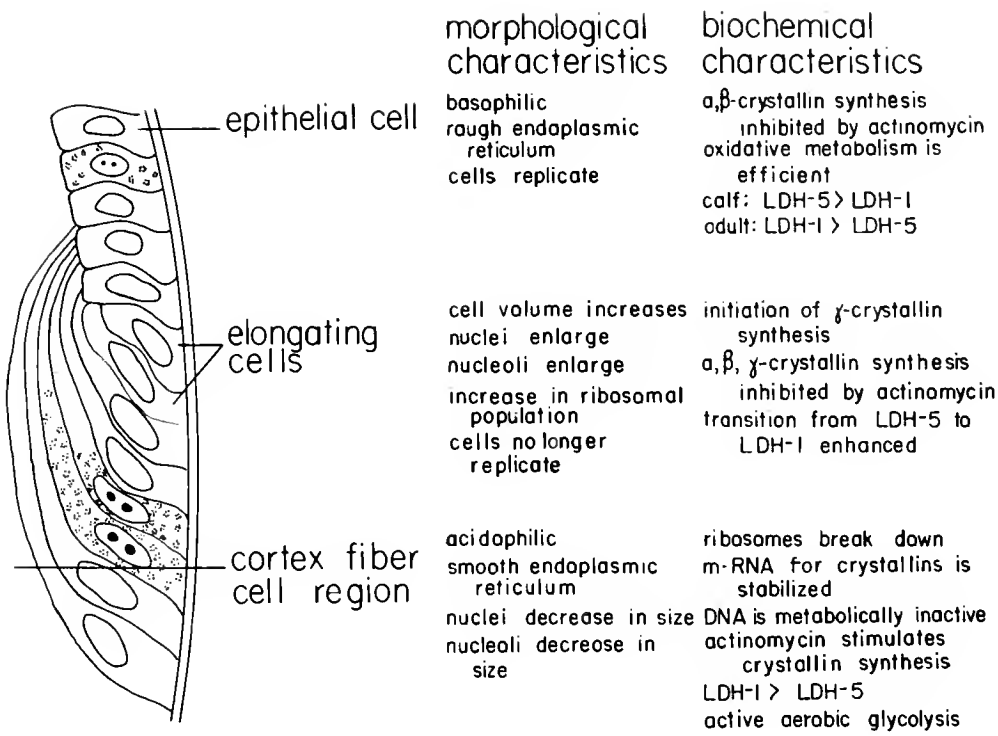

Fig. 2.

A diagramatic presentation of the region of cellular elongation in the vertebrate lens. The major morphological and biochemical characteristics associated with lens cell differentiation are listed and are discussed in detail in the text. (Fig. 2, J. Papaconstantinou, *Science*, In press; copyright 1966 by the American Association for the Advancement of Science.)

and γ-crystallins from DEAE columns are shown in Figs. 3B and 3C. The protein fractions from the cortex fiber cells (Fig. 3B) and from the nucleus fiber cells (Fig. 3C) of the adult lens were precipitated and further characterized by free boundary electrophoresis. Their electrophoretic mobilities are listed in Table I. The mobility of these fractions was used as a means of identification of the protein fractions eluted from the column.

At the time that these studies were initiated I was impressed by the mechanism of lens growth, especially, by the existence of many layers of fiber cells which are systematically laid down throughout the life of the animal. Theoretically, therefore, by peeling away the layers of fiber cells in an adult lens it should be possible to recover the cells formed at various ages. Actually, the fiber cells can be peeled off when the decapsulated lenses are placed in a buffered solution. The outer cortex fiber cells, for example, continue to peel off until the central, nucleus region is reached. The freed fiber cells can be separated from the nucleus fiber cells by decanting, and using this procedure for separating the fiber cells from the different lens regions, one could look for any chemical differences between cells that were laid down throughout the growth periods of the lens. Since the epithelial cells and elongating cells from the equatorial zone could be removed along with the lens capsule, we were now provided with a method for separating the lens cells into three groups: (a) the epithelial cells, (b) the newly formed cortex fiber cells and (c) the fiber cells of the nucleus region which had been laid down during the early life of the animal. These cells were homogenized in 0.005 M sodium phosphate buffer pH 7.0 and fractionated on DEAE-cellulose columns. Characteristic elution patterns for each of the regions were obtained as is shown in Figs. 3A, 3B and 3C. These are not pure fractions as can be seen from the electrophoresis data (Table I), but this

TABLE I

The Electrophoretic Mobility x 10^5 (cm^2 $volts^{-1}$ $sec.^{-1}$) of the lens α-, β- and γ-Crystallins from Adult Bovine Lens Cortex and nucleus fibers. The crystallins were fractionated on DEAE-cellulose columns. The peaks were precipitated and analyzed by free boundary electrophoresis.

		γ-crystallins		β-crystallins		α-, β-crystallins	α-crystallins		
DEAE fraction		$a_1 + a_2$	b	c	d	e	f	g	h
Cortex fiber cell proteins	Fast component	2.56	3.08	3.48	3.96	4.76	5.23	5.61	6.12
	Slow component	1.93	2.38	2.41	3.08	3.54	--	--	--
Nucleus fiber cell proteins	Fast component	2.22	--	3.13	3.98	4.30	5.04	5.10	5.22
	Slow component	1.72	--	2.93	2.94	3.37	--	--	--

method is quite good for separating the proteins into the α-, β- and γ-crystallin groups. The γ-crystallins, which are the proteins we are interested in for this discussion, are eluted cleanly from the column as peaks a_1, a_2 and *b* in the cortex fiber pattern (Fig. 3B) and as peak *a* in the nucleus fiber pattern (Fig. 3C).

POLLARD: What's the separation process? Is it on a column?

PAPACONSTANTINOU: This is a DEAE-cellulose column, using a stepwise elution system starting with 0.005 M phosphate buffer pH 7.0 and going to 0.02 M phosphate buffer pH 5.7. After this, further elution is achieved by increasing the ionic strength with NaCl. We have done linear gradients on this more recently and they are essentially the same. We've used two linear gradients: the first is a sodium phosphate gradient ranging from 0.005 M phosphate pH 7.0 to 0.02 M phosphate pH 5.7. With this, the γ- and β-crystallins are eluted from the column. Then the phosphate concentration is kept constant at 0.02 M pH 5.7 and a NaCl gradient is initiated. This results in the elution of the α-crystallins.

A comparison of these elution diagrams shows that the epithelial cells (Fig. 3A) contain only traces of γ-crystallins in comparison to the amounts found in adult cortex (Fig. 3B) and adult nucleus (Fig. 3C) fiber cells. Furthermore, it can also be seen that the γ-crystallins of the adult cortex and adult nucleus fiber cells are both qualitatively and quantitatively different with respect to their chromatographic properties on DEAE-cellulose columns. These observations indicate, firstly, that the γ-crystallins are proteins which are characteristic of the fiber cell and secondly, that γ-crystallins formed in fiber cells of young animals (cells found in the nucleus region of the adult lens) are chromatographically, and possibly chemically, distinct from γ-crystallins synthesized in fiber cells of older animals (cells found in the cortex region of the adult lens).

If the first proposal is correct, i.e., that γ-crystallins are proteins specific to the fiber cells, then epithelial cells from animals of all ages should lack these proteins. The elution pattern of proteins from epithelial cells of 3 month calf lenses (Fig. 4A) indicate that this is indeed the case. Although traces of γ-crystallins are detected by this procedure, the amount detected is significantly less than that detected in the fiber cells (Figs. 4B, 4C). In addition, the traces of γ-crystallins that are detected in the epithelial cells are due to the adherence of the elongating cells to the lens capsule. It is, we believe, in these elongating fiber cells, where the activation of γ-crystallin synthesis occurs. Thus, when we compare the elution patterns of proteins extracted from epithelial cells, cortex fiber cells and nucleus fiber cells of adult and calf lenses, we see that (a) at both ages the epithelial cells do not contain γ-crystallins and conclude that γ-crystallin synthesis is initiated during fiber cell formation in young and adult lenses. Similarly, it has been reported that γ-crystallin synthesis is associated with fiber

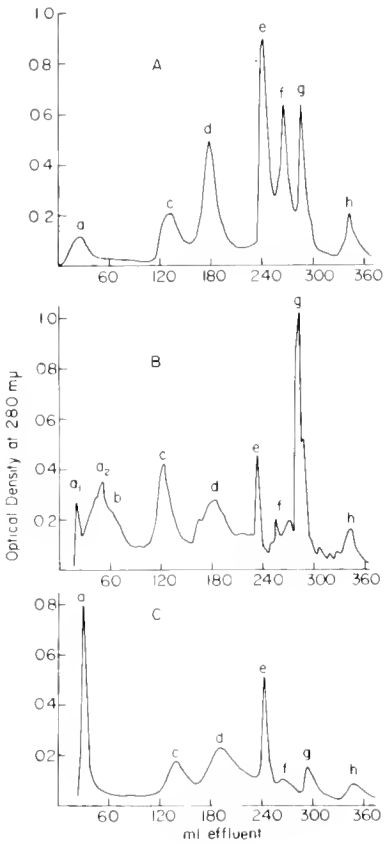

Fig. 3.

(A) Fractionation of soluble proteins from adult bovine lens epithelial cells. The cells were homogenized in 0.005 *M* phosphate buffer pH 7.0 and the homogenate was cleared by centrifuging at 10,000 x g for 10 min. The supernatant was dialyzed against 0.005 *M* phosphate buffer overnight. 74.0 mg of protein were added to 10 g of DEAE-cellulose; 60.29 mg protein were recovered at the end of the experiment. Buffers were added to the column in the following sequence: I. 50 ml 0.005 *M* sodium-phosphate pH 7; II. 50 ml 0.0075 *M* sodium-phosphate pH 6.5; III. 50 ml 0.01 *M* sodium-phosphate pH 6; IV. 75 ml 0.02 *M* sodium-phosphate pH 5.7; V. 50 ml 0.02 *M* sodium-phosphate pH 5.7 + 0.1 *M* NaCl; VI. 50 ml 0.1 *M* sodium-phosphate pH 5.7 + 0.1 *M* NaCl; VII. 50 ml 0.1 *M* sodium-phosphate pH 5.7 + 0.3 *M* NaCl. The fractions were collected in 3 ml aliquots. (B) Fractionation of soluble proteins from cortex fibers of the adult bovine lens. The elution sequence is the same as that shown above. 74.0 mg protein were placed on 10 g of DEAE-cellulose; 65.39 mg protein were recovered at the end of the experiment. (C) Fractionation of soluble proteins from nucleus fibers of adult bovine lens. The elution sequence is the same as that shown above. 73.92 mg protein were placed on 10 g of DEAE-cellulose; 43.77 mg protein were recovered at the end of the experiment. (Fig. 1, J. Papaconstantinou, *Biochim. Biophys. Acta 107*, 81, 1965; reproduced with permission of Elsevier Publishing Company.)

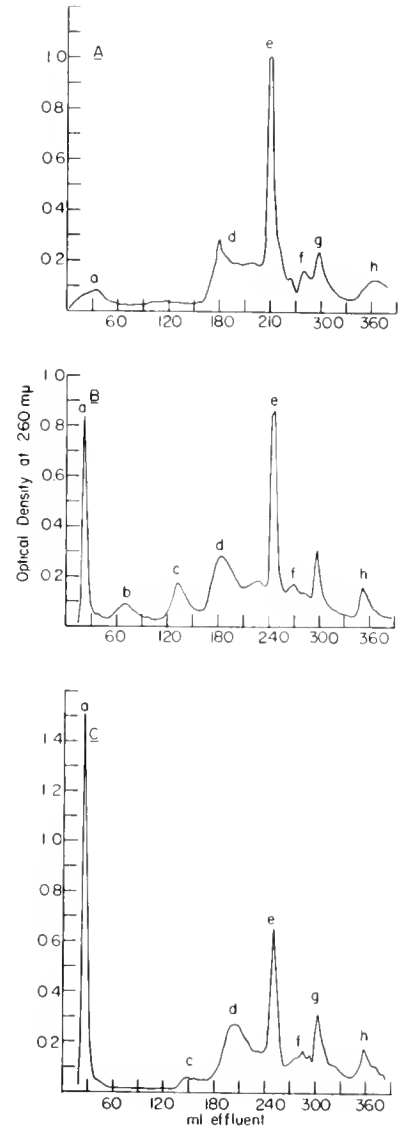

Fig. 4.

(A) Fractionation of soluble proteins from epithelial cells of calf lenses. 48.24 mg protein were placed on 10 g of DEAE-cellulose; 41.63 mg protein were recovered at the end of the experiment. Buffers were added to the column in the sequence described in Fig. 3. (B) Fractionation of soluble proteins from calf cortex fibers. 49.80 mg protein were placed on 10 g DEAE-cellulose; 51.40 mg protein were recovered at the end of the experiment. The elution sequence is the same as that described above. (C) Fractionation of soluble proteins from calf nucleus fibers. 50.23 mg protein were placed on 10 g of DEAE-cellulose; 49.90 mg were recovered at the end of the experiment. The elution sequence is the same as that described above. (Fig. 2, J. Papaconstantinou, *Biochim. Biophys. Acta 107*, 81, 1965; reproduced with permission of Elsevier Publishing Company.)

cell formation in the regenerating salamander lens (10).

In my second proposal, I stated that the variation in DEAE column properties between adult cortex and adult nucleus γ-crystallins imply distinct differences exist between the γ-crystallins of the nucleus region and cortex region of the adult lens. Since the adult nucleus fibers are cells which were formed during the earlier period of lens growth, these regional differences in γ-crystallins of the adult lens (Figs. 3B and 3C) may be due to amino acid differences in the γ-crystallins formed by fiber cell differentiation at differentiation at different ages. Thus, the γ-crystallins from lenses of younger animals (embryos and young calves) should have the same chromatographic properties as γ-crystallins from the nucleus fibers of an adult lens. Evidence for this is presented by the elution patterns for proteins from calf cortex (Fig. 4B), calf nucleus (Fig. 4C) and embryonic lenses (Fig. 5), which show that the γ-crystallins in the fiber cells of these younger lenses are chromatographically similar to the

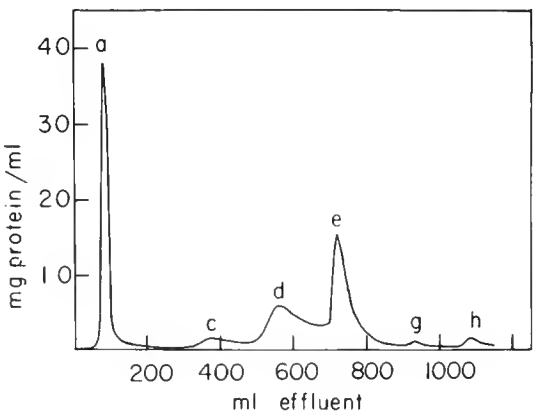

Fig. 5.

Fractionation of the soluble proteins from the combined lenses of 115 day and 130 day embryos. 426.24 mg protein in a volume of 7.4 ml were added to a DEAE-cellulose column (2 cm x 10 cm). 302.9 mg protein were recovered at the end of the experiment. The elution sequence and volume of buffers used are as follows: I. 100 ml 0.05 *M* sodium-phosphate pH 7; II. 200 ml 0.0075 *M* sodium-phosphate pH 6.5; III. 150 ml 0.01 *M* sodium-phosphate pH 6; IV. 200 ml 0.02 *M* sodium-phosphate pH 5.7; V. 500 ml 0.02 *M* sodium-phosphate pH 5.7 + 0.1 *M* NaCl; VI. 150 ml 0.1 *M* sodium-phosphate pH 5.7 + 0.1 NaCl; VII. 150 ml 0.1 *M* sodium-phosphate pH 5.7 + 0.3 NaCl. The fractions were collected in 10 ml aliquots. (Fig. 3, J. Papaconstantinou, *Biochim. Biophys. Acta 107*, 81, 1965; reproduced with permission of Elsevier Publishing Company.)

γ-crystallins of the adult nucleus fibers (Fig. 3C). Furthermore, it can be seen that only in the elution pattern of the calf cortex fibers (Fig. 4B), where the predominating γ-crystallins are of the "embryonic type", are there indications of the appearance of the types of γ-crystallins observed in the adult cortex fiber cells, i.e., peaks a_2 and *b* (Fig. 3B). The patterns for calf nucleus fiber cell proteins and embryonic lens proteins show a complete absence of adult cortex fiber type γ-crystallins.

In view of the differences in chromatographic properties of the γ-crystallins attempts were made to obtain further evidence for more distinct differences between the embryonic and adult γ-crystallins. Further purification of adult cortex, adult nucleus and embryonic γ-crystallins was achieved by DEAE-cellulose fractionation using tris buffer ranging in pH from 10 to 7 (Fig. 6). In each case the γ-crystallins were resolved into 4 major proteins. Our observations are similar to those of Bjork (11) who, through the use of alternative procedures of fractionation, was able to resolve the γ-crystallins into 4 distinct fractions. The purified γ-crystallins from each of these fractions were concentrated and their relative mobilities were determined by paper electrophoresis. The electrophoretic patterns (Figs. 7, 8) show that the γ-crystallins from embryonic and adult nucleus fibers have the same mobility, whereas the γ-crystallins of the adult cortex have a different mobility. These data are in agreement with the preliminary observations on DEAE-columns.

From these observations it can be concluded that (a) γ-crystallin synthesis is initiated during fiber cell formation and is associated with this specific stage of lens cell differentiation and (b) that the γ-crystallins synthesized during embryonic and early post-natal fiber cell differentiation are electrophoretically distinct from those synthesized in the fibers of the adult lens. Thus, the type of γ-crystallin synthesized depends on the age of the animal or possibly the rate at which fiber cells are laid down (5, 6).

With respect to the initiation of γ-crystallin synthesis we have an example of gene activation at the molecular level, and one question which concerns us now is whether the activation of γ-crystallin synthesis is intimately associated with the genetic regulation of the morphological changes in the cell. Furthermore, since fiber cell formation involves the transition of a replicative cell to a non-replicative cell, is γ-crystallin in any way associated with this aspect of lens cell differentiation? We are

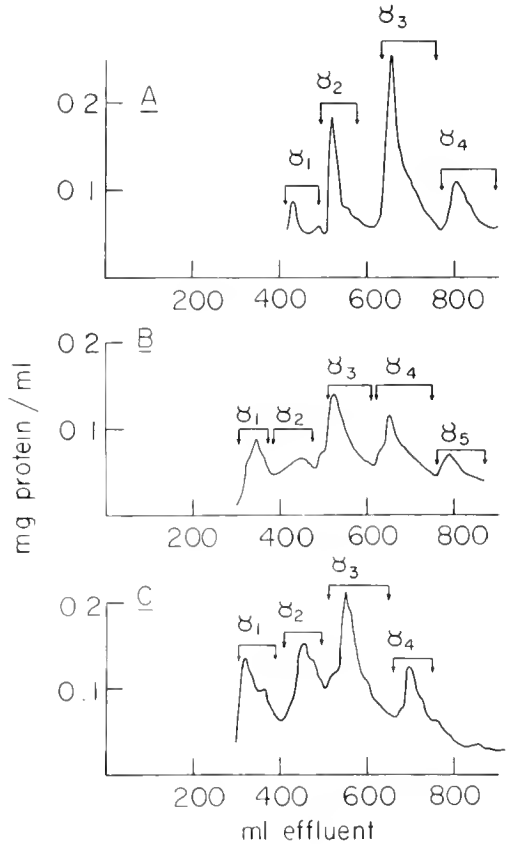

Fig. 6.

The fractionation of γ-crystallins from adult cortex, adult nucleus and embryonic lenses. The proteins, which had been stored as an ammonium sulfate precipitate were spun down and dissolved in 0.01 *M* Tris, pH 10. Ammonium sulfate was eliminated by dialysis against 0.01 *M* Tris. The protein solution was placed on a DEAE-cellulose column and was eluted from the column by the stepwise addition of Tris-HCl buffers in the following order: I. 150 ml 0.01 *M* Tris, pH 10; II. 150 ml 0.02 *M* Tris, pH 9; III. 150 ml 0.04 *M* Tris, pH 8.6; IV. 150 ml 0.06 *M* Tris-HCl, pH 8.2; V. 150 ml 0.08 *M* Tris-HCl, pH 7.6; VI. 150 ml 0.1 *M* Tris-HCl, pH 7.2. In each fractionation the column size was 17 mm diameter x 20 cm height; 5 ml aliquots were collected. (Fig. 4, J. Papaconstantinou, *Biochim. Biophys. Acta 107*, 81, 1965; reproduced by permission of Elsevier Publishing Company.)

presently involved in experiments designed to determine whether epithelial cells *in vivo* and in tissue culture can be induced to synthesize γ-crystallins and at the same time retain both their epithelial cell structure and their ability to replicate.

B. The loss of LDH-5 isozyme synthesis in lens cell differentiation: gene repression.

I would like to present you with another example of differential gene action associated with fiber cell differentiation as well as with the aging of the epithelial cells, i.e., the specific repression of one of the lactate dehydrogenase isozymes. These experiments were carried out in collaboration with Mr. James A. Stewart (12, 13).

Lactate dehydrogenase isozymes have been shown to occur in many vertebrate tissues in 5 electrophoretically distinct forms, and to vary in activity during embryonic and post-embryonic development (14-19). In addition, it is now well established that all 5 isozymes are composed of 4 protein subunits and that only the extreme cathodal (LDH-5) and anodal (LDH-1) forms of these enzymes are homogeneous with respect to their subunits. Furthermore the subunits of LDH-1 do not have the same amino acid composition as the subunits of LDH-5. Thus, by dissociation and reassociation of the subunits in a mixture of LDHs-1 and 5, all 5 isozymes can be formed (20). These experiments show that LDH's-2, 3 and 4 are composed of combinations of LDH-1 and 5 subunits. Since there is now good evidence that the synthesis of LDH-1 and LDH-5 subunits is genetically regulated (21), we felt that any alterations in the isozymic patterns during fiber cell differentiation would be another indication of the differential regulation of protein synthesis which in the lens could be localized to a very specific stage of cellular differentiation, namely, the differentiation of an epithelial cell to a fiber cell.

Our electrophoretic analyses of the lactate dehydrogenase isozymes show that the epithelial cells of the adult lens and calf lens have five forms of the enzyme. Typical isozymic patterns of calf and adult epithelial and fiber cells are diagrammatically presented in Fig. 9. Concentrating on the epithelial cell diagrams alone, a comparison of the patterns from calf and adult cells shows that a change occurs from predominantly cathodal forms to predominantly anodal forms. These data show that there is a transition of epithelial cell LDH isozyme activity during the post-natal aging of these cells. This is a very interesting change since the epithelial cells of the lens, during embryonic, early post-natal and adult life, carry out the same functions, i.e., to either replicate for the formation of more epithelial cells or to differen-

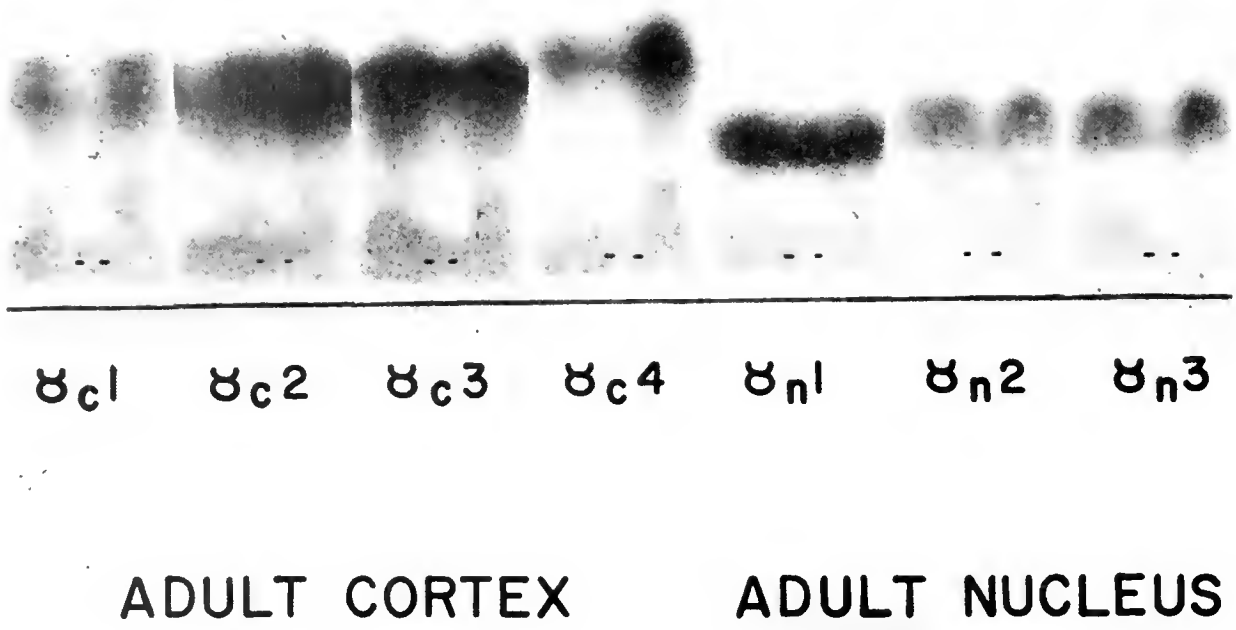

Fig. 7.

Electrophoretic analysis of the adult cortex and nucleus γ-crystallins. Electrophoresis was carried out in 0.5 *M* Tris-0.021 *M* EDTA-0.075 *M* boric acid, pH 8.9, at constant voltage (5.8 V/cm) for 17 hrs. The γ-crystallins tested were prepared by DEAE-fractionation, precipitated in ammonium sulfate and redissolved in 0.05 *M* Tris. (Fig. 5, J. Papaconstantinou, *Biochim. Biophys. Acta* 107, 81, 1965; reproduced with permission of Elsevier Publishing Company.)

tiate into fiber cells. The only functional variation between the epithelial cells of young and adult lenses is their mitotic activity, which is greater during the early periods of lens growth (22-25). As the lens reaches its maximum size in the adult, mitotic activity decreases. It is during this decrease in mitotic activity that the major transformation from LDH-5 to LDH-1 occurs. On this basis it might be proposed that this isozymic transition is associated with a slowing down of mitotic activity as well as other metabolic functions. An example which might be considered similar to the aging of the lens epithelial cells has been reported by Dawson, *et al.*, (18). They have found that more LDH-1 and less LDH-5 are found in samples of human muscle obtained from elderly people and that the highest concentration of LDH-5 subunits is found in muscle from healthy adult males.

Now I would like to consider our observations on the LDH isozyme patterns during the differentiation of epithelial cells to fiber cells. In addition to the changes in the epithelial cells alone we found that in both the calf and adult lens pronounced changes occur during the differentiation of the fiber cell. This is the final stage of lens cell differentiation and results in the transition from a replicative cell to a non-replicative cell. During this stage of differentiation LDH-1 persists. In the calf, the fiber cells contain 5 detectable LDH's in which LDH-1 is predominant. In the adult, the fiber cells contain essentially just LDH-1 although small amounts of LDH-2 are detectable. On the basis that the synthesis of subunits of LDH's-1 and 5 are genetically regulated, the complete loss of LDH's-3, 4 and 5 in the adult lens fiber cell and the intermediate trends toward this loss in the calf cells might be attributed to the suppression of LDH-5 subunit synthesis which would decrease

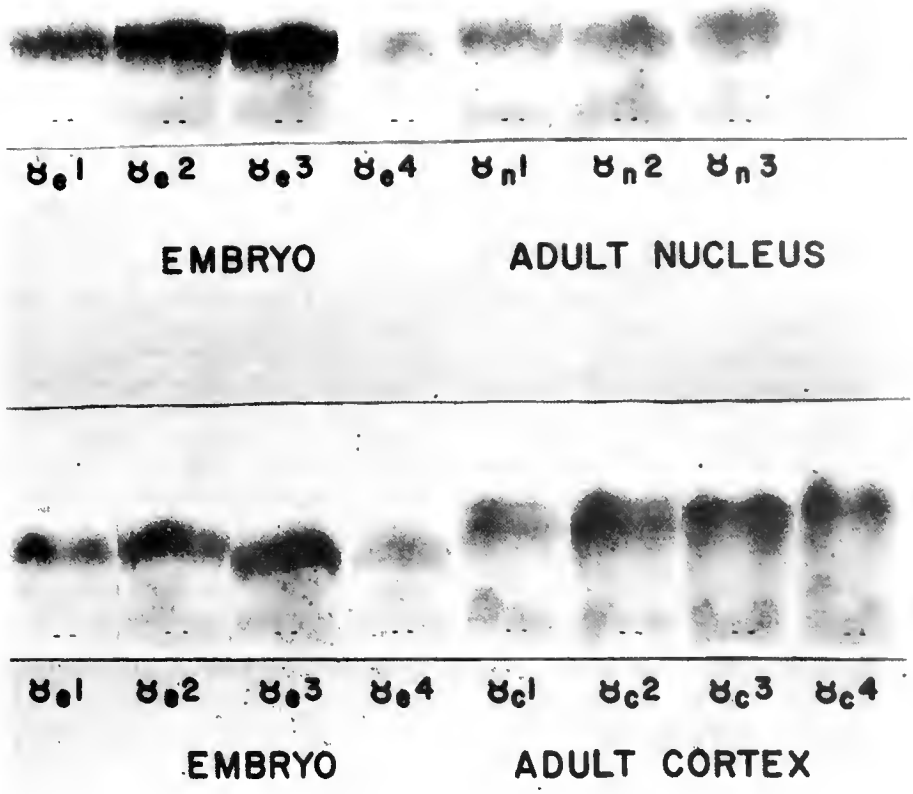

Fig. 8.

Electrophoretic analysis of γ-crystallins from adult and embryonic bovine lenses. The conditions of electrophoresis are the same as described in Fig. 7. (Fig. 6, J. Papaconstantinou, *Biochim. Biophys. Acta 107*, 81, 1965; reproduced with permission of Elsevier Publishing Company.)

their availability for recombination with LDH-1 subunits.

We interpret these data to indicate that during the aging of lens epithelial cells (calf to adult) there is a regulation of LDH subunit synthesis such that there is a greater decrease in the synthesis of LDH-5 subunits than LDH-1 subunits. Furthermore, this tendency for the persistence of LDH-1 becomes more pronounced during the differentiation of the epithelial cell to the fiber cell in both the calf and adult lens. The extreme case is seen in the adult cortex fiber cells where LDH-1 is essentially the only one of the 5 isozymes remaining. Finally, we would like to correlate this with the replicative ability of the cell. When these cells, which retain their ability to replicate, reach a phase analogous to the stationary phase of a logarithmic growth cycle the synthesis of LDH-1 subunits is favored. This is also the case when the cells reach a stage of differentiation in which they have lost their replicative capacity (the fiber cells).

C. LDH isozymes and lens carbohydrate metabolism

I would like to digress from the main theme of my talk for one moment to correlate the LDH isozyme data just presented with the metabolic activity of the lens epithelial cells and fiber cells. A possible role of the regulation of carbohydrate metabolism in skeletal and heart muscle has been attributed to the persistence of different LDH isozymes in these tissues. It has been observed that LDH-1 is the predominant isozyme in tissues exhibiting high rates of oxidative metabolism, such as embryonic and

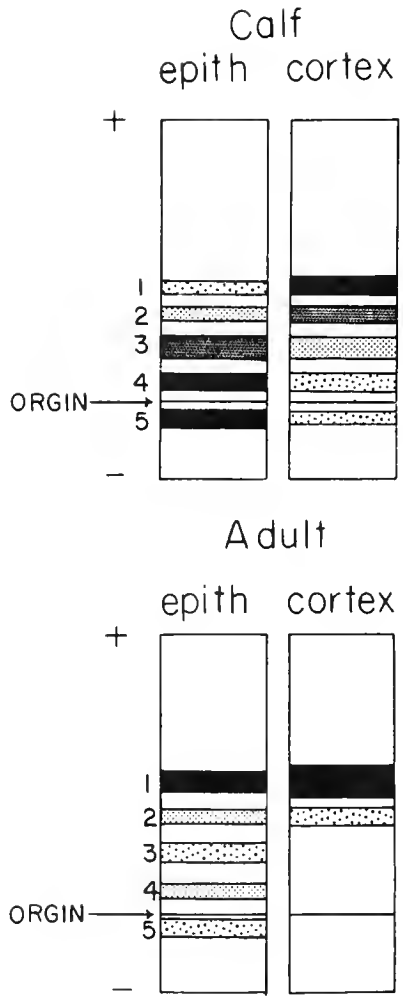

Fig. 9.

A diagramatic presentation of the LDH isozyme patterns for calf and adult lens epithelial cells and fiber cells. (Fig. 7, J. Papaconstantinou, *Science*, in press; copyright 1966 by the American Association for the Advancement of Science.)

adult heart tissue of mouse and chicken, while LDH-5 is the predominant form in tissues that can function under conditions of oxygen debt, such as adult skeletal muscle. Furthermore, it has been shown that LDH-1 is more sensitive to inhibition by high pyruvate concentrations than LDH-5 (14, 15, 17). On the basis of these observations it has been postulated that in highly oxidative tissues such as the heart, the level of lactic acid is regulated, i.e., kept at a low level, because of the sensitivity of LDH-1 to pyruvate. This hypothesis is further borne out by the fact that skeletal muscle, which is capable of tolerating a greater variation in oxygen tension than heart muscle, contains more active LDH-5, the isozyme which shows less sensitivity to substrate inhibition.

Let us now consider the metabolic properties of the lens cells. Wanko and Gavin (25, 26) reported that the epithelial cells have relatively more mitochondria than the fiber cells and that the population of epithelial cell mitochondria is significantly decreased after fiber cells are formed. Thus, metabolically the epithelial and fiber cell differs significantly in that the former cell type exhibits a greater degree of aerobic, oxidative metabolic pathways. Epithelial cells have been shown to have higher levels of cytochrome c, greater succinate dehydrogenase activity, and more active mitochondria (27). Fiber cells, on the other hand, have been shown to have a greater degree of aerobic glycolysis (28). Furthermore, it has been shown that the most efficient production of ATP from ADP in calf cortex fibers occurs with fructose-1, 6-diphosphate as substrate (29). Krebs cycle enzymes are detectable in fiber cells, but their activity is significantly less than that found in the epithelial cells. All of these observations indicate that a major metabolic difference between epithelial and fiber cells is in their respiratory and glycolytic activity.

Taking the metabolic properties of lens cells into account it would appear from the work on heart and skeletal muscle LDH that the lens fiber cells should retain LDH-5. Our data have shown the opposite, i.e., that the fiber cells retain LDH-1. In addition, even though LDH-1 is retained, high lactic acid levels are maintained by these cells.

Several factors such as oxygen tension, intracellular pools of metabolic intermediates and cofactors, and predominating pathways of carbohydrate metabolism have been postulated to play an important role in the type of LDH isozymes retained by a specific tissue (30, 31). Recent work on the LDH isozymes in cultured chick heart muscle cells has shown that after 6 days in culture LDH-5 is the predominant form (30). Prior to being placed in culture these cells have predominantly LDH-1. In fact, chick heart cells have been shown to retain LDH-1 throughout embryonic and post-embryonic life. Thus, under tissue culture conditions a new phase of LDH isozyme distribution, not previously experienced by these cells, is developed. This predominance of LDH-5 was significantly slowed down when placed under conditions of high oxygen tension or when Krebs cycle intermediates are added to the culture medium.

These observations cannot, however, explain the persistence of LDH-1 in the lens fiber cells, since the oxygen tension in the lens is lower than that in the blood and the pathways of oxidative metabolism are practically negligible. Even under these conditions the highly glycolyzing fiber cells retain LDH-1 thus showing that within this tissue some other factor or factors related to the replicative capacity must also be considered in explaining the regulation of LDH subunit synthesis.

I have now come to the end of our observations on the regulation of synthesis of tissue specific proteins associated with a specific stage of cellular differentiation. Our data have shown that the synthesis of γ-crystallins is specifically associated with the differentiation of the epithelial cell to the fiber cell. Thus, the α- and β-crystallins are structural proteins of the epithelial cell and the α-, β- and γ-crystallins are structural proteins of the fiber cell. At the beginning of my talk I described some cytological changes which occur in elongating epithelial cells such as an enlargement of the nucleus and nucleoli and an increase in the ribosomal population. These observations are indicative of an increase in protein synthesis and may be associated with the initiation of γ-crystallin synthesis.

The lactate dehydrogenases on the other hand have shown us the simultaneous "turning off" of a specific protein which is associated not only with fiber cell formation, but also with the aging and replicative activity of the cell. Thus, the ability of the cell to regulate LDH subunit synthesis in the absence of morphological changes brings out a significant difference between the regulation of γ-crystallin synthesis and LDH subunit synthesis. The γ-crystallins are highly tissue specific proteins whose function may be essentially involved in the structure of the lens whereas the LDHs are widespread and are essential for metabolic activity. In both cases, differential gene action is required. Whether these regulatory mechanisms are similar must await further experimentation.

IV. The Role of Nucleic Acids in Lens Fiber Cell Differentiation

A. The status of m-RNA in differentiating lens cells: the stabilization of m-RNA

It has recently been shown that the synthesis of specific proteins such as hemoglobins (32), feather keratins (33) and lens crystallins (34-38) occurs on relatively long lived m-RNA templates. These long lived messengers are found in highly differentiated cells and are involved in the synthesis of proteins specific for these cells. Bacterial m-RNA for example, which is considered to be short lived has a half-life of 2 minutes (39), while the half-life of m-RNA for feather keratin synthesis has been reported to be longer than 24 hours (33). At present, the only way to show stable m-RNA is through the insensitivity of a protein-synthesizing polysomal unit to actinomycin D, and all the cases described so far are based on the observation that protein synthesis continues long after RNA synthesis has been halted by actinomycin. On the basis of these preliminary observations, it appears that the stability of m-RNA is a very important feature of the differentiated cell in which a large percentage of the proteins synthesized are tissue specific proteins. Although many tissue specific proteins appear in the initial stages of tissue differentiation, the basic question we would like to consider is whether these proteins are synthesized on "pre-existing" stable templates or whether there is a progressive transition from an actinomycin sensitive to an actinomycin insensitive period of protein synthesis.

In a series of experiments carried out by Mr. James A. Stewart, Dr. Paul V. Koehn and myself (36-38), we attempted to determine whether (a) the lens crystallins of the epithelial cells and fiber cells are synthesized on long lived or short lived messenger templates and (b) if there is some period of lens cell differentiation in which the m-RNA passes from a stage of actinomycin sensitivity to actinomycin insensitivity, thus associating the stabilization of m-RNA to a specific stage of cellular differentiation.

In these experiments, intact bovine calf lenses were incubated in the presence of C^{14}-amino acids with and without actinomycin D (10 μg/ml). The epithelial and fiber cells were separated and the crystallins from each cell type were fractionated on DEAE-cellulose columns. An elution diagram of the separation of α-, β- and γ-crystallins of untreated and actinomycin treated epithelial cells is shown in Figs. 10 and 11. The incorporation of amino acids into these proteins is also shown. It can be seen that incorporation of amino acids into epithelial cell crystallins could be extensively inhibited by actinomycin. Both elution diagrams show similar protein patterns. The specific

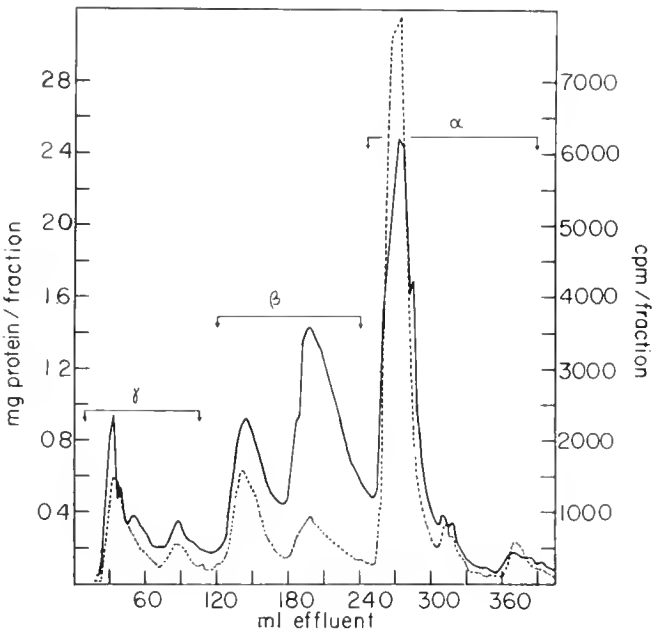

Fig. 10.

The fractionation of calf lens epithelial cell proteins on DEAE-cellulose after incubation in ^{14}C-algal hydrolysate (amino acids) for 2 hours at 37°C. The elution system is the same as that described for Fig. 3. The solid lines denote total proteins (mg) per 3 ml fraction. The dotted lines denote total counts per minute per 3 ml fraction. (Fig. 8, J. Papaconstantinou, *Science*, in press; copyright 1966 by the American Association for the Advancement of Science.)

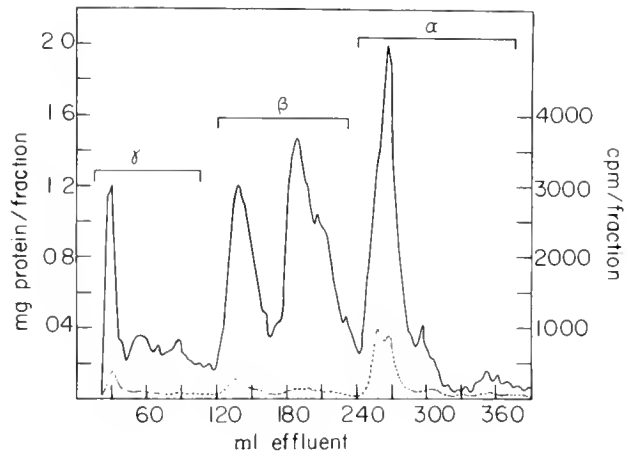

Fig. 11.

The fractionation of calf lens epithelial cell proteins on DEAE-cellulose after incubation in ^{14}C-algal hydrolysate (amino acids) with 10 μg/ml actinomycin D. The experimental conditions are the same as those described in Fig. 10. (Fig. 9, J. Papaconstantinou, *Science*, in press; copyright 1966 by the American Association for the Advancement of Science.)

activity data (Table II), however, show that incorporation of amino acids into α-crystallins was inhibited by 71%, β-crystallins by 83% and γ-crystallins by 80%.

The same experiments were performed with the lens fiber cells. Elution patterns of α-, β- and γ-crystallins of cortex fiber cells incubated in the absence and in the presence of actinomycin D are seen in Figs. 12 and 13, respectively. The incorporation of amino acids into these proteins is also shown and, again, both patterns are essentially identical with respect to the distribution of the crystallins. The incorporation of amino acids into the crystallins, however, is significantly greater in the actinomycin treated cells. A comparison of the specific activity of the α-, β- and γ-crystallins from control and actinomycin treated lenses shows that there is a significant stimulation of protein synthesis by the antibiotic which ranges from 66% for the β-crystallins to 103% for the α-crystallins (Table II).

A comparison of the specific activity of actinomycin treated cells shows an 85% inhibition of γ-crystallin synthesis in the elongating epithelial cells and a 68% stimulation of this same group of proteins in the fiber cells. Thus, at the time of γ-crystallin appearance the synthesis of this protein, as well as of the α- and β-crystallins, is still sensitive to inhibition by actinomycin D, whereas in the completed fiber cell the synthesis of these same proteins is stimulated. The mechanism by which actinomycin D stimulates protein synthesis is unknown. The mechanisms which have been proposed for this effect are as follows: first, the stimulation might be attributed to the availability of more ATP for protein synthesis as a result of the inhibition of RNA synthesis by actinomycin (40). Thus, in the fiber cell the ATP normally used for RNA synthesis could be channeled into the synthesis of the proteins being formed on stable RNA templates.

POLLARD: Quite apart from that, however, if you're just going to use one protein, aren't you only using the t-RNA more efficiently on that one protein when you shut off the others? If all you're doing is just using one protein and if you've got a long-lived message, isn't there every reason why it would go up?

PAPACONSTANTINOU: Yes, that's right; if that's the explanation for it.

TABLE II

The Effect of Actinomycin D on Lens Protein Synthesis in Calf Lens Epithelial and Fiber Cells.

Epithelial Cells				Cortex Fiber Cells			
	cpm/mg Protein in						
	Control	Act. D	% Inhibition		Control	Act. D	% Stimulation
α-crystallins	1980	572	71	α-crystallins	340	690	103
β-crystallins	963	166	83	β-crystallins	99	165	66
γ-crystallins	1590	314	80	γ-crystallins	235	396	68

POLLARD: How can you avoid having that happen if the t-RNA is there? It seems to me you've got to have some stimulation if there is a long-lived message present.

GROSS: We reported, in 1962, stimulation by actinomycin in the sea urchin and we suggested that it was the sparing of ATP.

PAPACONSTANTINOU: Tomkins and his coworkers have recently presented their observation on the stimulatory effect of actinomycin on liver enzyme activity. They have shown that the induction and early periods of enzyme synthesis are inhibited by actinomycin whereas after a given period of time enzyme activity is stimulated by actinomycin. They believe that at the time these enzymes are stimulated by actinomycin their m-RNA is stable and a repressor is inhibiting further synthesis of this m-RNA. The stimulation occurs only when actinomycin inhibits further synthesis of the repressor. Pollock has also shown a stimulation of penicillinase by actinomycin in *B. subtilis*. He also showed that β-galactosidase is not stimulated. One of his explanations for this is a difference in binding of actinomycin to the bacterial genome, thus explaining the differential sensitivity of these two enzymes to actinomycin. He proposes that this differential binding of actinomycin may be a function of the GC content of the individual genes.

GROSS: That's a fine idea if you can show that all the RNA synthesis is lost. Pollock used very low levels of the drug and he did not present fully convincing evidence that he had shut down all synthesis of template RNA.

PAPACONSTANTINOU: Well, it didn't matter whether he shut it down or not. He showed he got a stimulation of the penicillinase. If he'd gotten no effect I could accept that argument; but he got an effect.

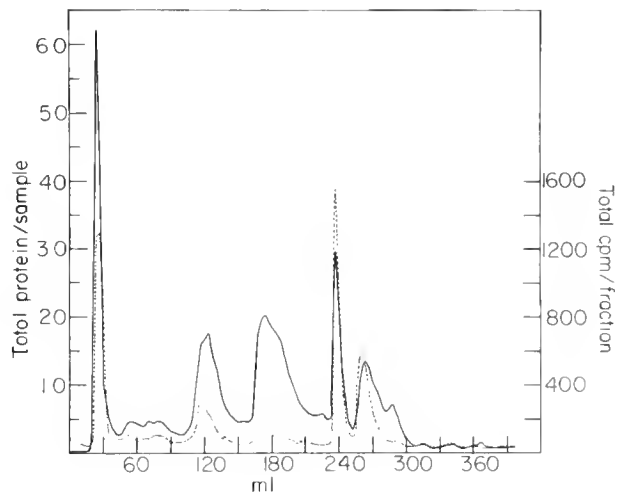

Fig. 12.

The fractionation of calf lens cortex fiber cell proteins. The experimental conditions are the same as those described for Fig. 10. (Fig. 10, J. Papaconstantinou, *Science*, in press; copyright 1966 by the American Association for the Advancement of Science.)

GROSS: He might have had the penicillinase messenger still coming out, but the outflow of others seriously depressed; so that you're synthesizing the proteins from templates that remain at a selectively higher rate.

PAPACONSTANTINOU: Right, What you're essentially saying is that the penicillinase region may not have as high a GC content as some of the other regions.

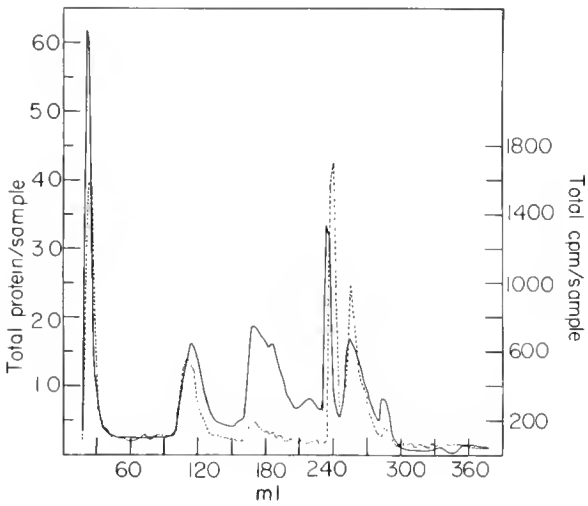

Fig. 13.

The fractionation of calf lens cortex fiber cell proteins incubated with ^{14}C-algal hydrolysate and 10 μg/ml actinomycin D. The experimental conditions are the same as described for Fig. 10. (Fig. 11, J. Papaconstantinou, *Science*, in press, copyright 1966 by the American Association for the Advancement of Science.)

GROSS: Well, whether its GC or not, they're not as sensitive.

PAPACONSTANTINOU: Right. I'm just presenting all these ideas which have come out in the literature. They're not my ideas, and I'm just trying to fit some of our data into any one or all of these as we go along. However, I think the evidence does seem to indicate that we can pinpoint the stage at which the messenger RNA becomes stabilized, and that is at the time when the epithelial cells are starting to elongate. The γ-crystallin is still sensitive to actinomycin at that stage, but when they've finally elongated, it's no longer sensitive. This holds true, also, for the α- and β-crystallins. The period of stabilization seems to fall in concurrently with the breakdown of ribosomes and the decrease in the size of the nucleus and nucleoli.

Secondly, this stimulation might be attributed to inhibition of the synthesis of a repressor protein by actinomycin (41). It has been shown that the actinomycin D stimulation of tryptophan pyrrolase and tyrosine transaminase occurs after these enzymes have been induced by hydrocortisone, when their m-RNA is relatively stable. By inhibiting the m-RNA responsible for the synthesis of repressor protein the level of this repressor is decreased and a stimulation of these enzymes results. Finally, the lens epithelial cells are essential for the active transport of nutrients into the lens fiber cells. It is, therefore, possible that actinomycin alters these properties such that there is an increase in the transport of amino acids into the fiber cell layer, resulting in a stimulation of protein synthesis on stable RNA templates.

B. Ribosomal breakdown in lens fiber cells

In this final phase of my talk I would like to describe a phenomenon, again associated with fiber cell differentiation which may explain the reduced rate of protein synthesis observed in the final cell. I shall start by reiterating that Eguchi and Karasaki (3, 4) showed by electron microscope studies that during fiber cell formation, in the elongating epithelial cell there is an increase in ribosomes, whereas in the completed fiber cell the ribosomal population is decreased. We feel that we have been able to show essentially the same thing in our chemical analyses of the ribosomal RNA in the fiber cell. In these studies we have used methylated albumin columns (42) to fractionate nucleic acids from lens epithelial cells and fiber cells and to detect any qualitative or quantitative differences that may occur in the nucleic acids of these cells. The fractionation procedure involved the use of a linear NaCl gradient in 0.05 M Na-phosphate buffer pH 6.8. An elution pattern of the epithelial cell nucleic acids is shown in Fig. 14. The t-RNA (peak A) is eluted between 0.4 M - 0.6 M NaCl; DNA (peak B) is eluted between 0.6 M - 0.8 M NaCl; and ribosomal RNA (peak C) is eluted between 0.8 M - 1.0 M NaCl. This sequence of elutions compares well with a similar elution system used to fractionate t-RNA, DNA and ribosomal RNA from *E. coli* (43). A pattern for the RNA extracted from fiber cells is superimposed over the epithelial cell pattern to facilitate comparisons between them. It can be seen that there are striking differences between the two patterns: (a) the pattern for fiber cell RNA shows a significantly larger amount of material eluted in peak A (t-RNA) with respect to the amount of material in the ribosomal RNA (peak C); (b) in addition, there is a sharp decrease in the DNA (peak B) of the fiber cell pattern. The quantitative differences between t-RNA and R-RNA of epithelial and fiber cells are better seen in the patterns of Fig. 15. Phenol extracted nucleic acids from epithelial and fiber cells were DNase treated to remove DNA, prior to fractionation on methylated albumin columns. The DNA (peak B) is completely lost,

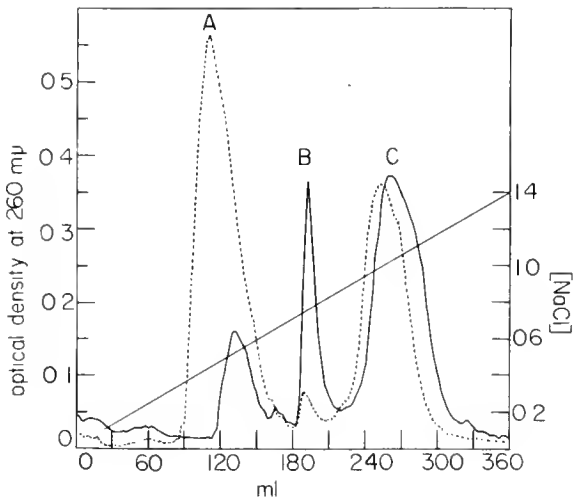

Fig. 14.

The fractionation of phenol extracted nucleic acids from calf lens epithelial cells (———) and calf cortex fiber cells (------) on methylated albumin columns (MAK). A total of 35.4 O.D. $_{260}$ units from epithelial cells were placed on the column; 45.0 O.D. $_{260}$ units from the fiber cells were placed on the column. The nucleic acids were eluted with a linear salt gradient ranging from 0.2 *M* to 1.4 *M* NaCl in 0.05 *M* sodium-phosphate pH 6.8. (Fig. 12, J. Papaconstantinou, *Science*, in press; copyright 1966 by the American Association for the Advancement of Science.)

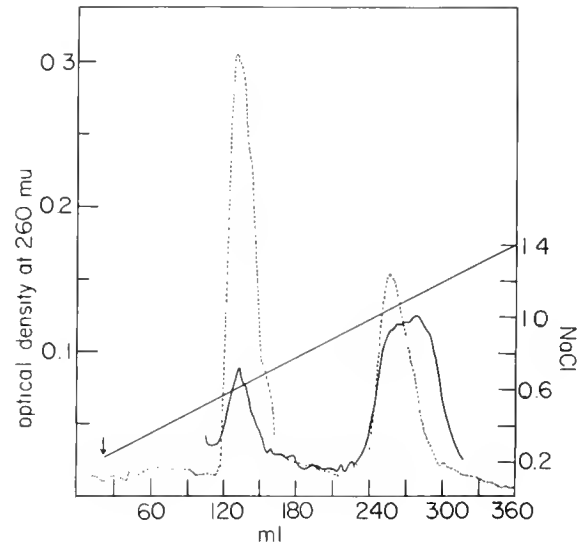

Fig. 15.

The fractionation of DNase-treated phenol-extracted nucleic acids from calf lens epithelial cells (———) and calf cortex fiber cells (------). The conditions of fractionation are exactly as described in Fig. 14. (Fig. 13, J. Papaconstantinou, *Science*, in press; copyright by the American Association for the Advancement of Science.)

and now the epithelial and fiber cell patterns are almost alike except for the quantitative differences between peaks A and C (Fig. 15).

Our next step was to determine whether the RNA of peak A is t-RNA or a mixture of t-RNA and a ribosomal RNA breakdown product. (This mixture will be referred to below as total soluble RNA). In his studies of the RNA fractions from *E. coli* Midgely showed that t-RNA and ribosomal RNA could be separated on DEAE columns using 0.05 M tris pH 7.4 with an increasing NaCl gradient (44). We carried out a similar fractionation to determine whether peak A is a mixture of t-RNA and ribosomal RNA. We used this procedure to determine whether the total soluble RNA from lens epithelial and fiber cells could be resolved into two fractions. In one experiment, phenol-extracted RNA was first placed on a sucrose gradient to eliminate the ribosomal RNA. The material remaining at the top of the gradient was dialyzed against tris buffer and was then fractionated on a DEAE column. The elution diagrams in Figs. 16A and 16B show a fiber cell and epithelial cell pattern respectively. Firstly, it can be seen from the OD_{260} readings that there is a small amount of RNA eluted by 0.5 M NaCl, which corresponds to the region where bacterial t-RNA is eluted. Another larger fraction is eluted by 0.7 - 0.8 M NaCl, which corresponds to the region where bacterial ribosomal RNA is eluted. Secondly, it can be seen that there is a significant increase in the ribosomal RNA fraction in the fiber cell pattern. In another experiment the total soluble RNA from calf cortex fiber cells was separated from ribosomal RNA by fractionation on a MAK column. The soluble RNA fractions (peak A) were pooled, dialyzed against tris buffer and fractionated on DEAE-cellulose. This fractionation is shown in Fig. 16C. It can be seen that this elution pattern is identical to that obtained for the total soluble RNA from the sucrose gradient (Fig. 16A). These preliminary data indicate that the increase in total soluble RNA (peak A) of the fiber cell (Figs. 14, 15) is due to the accumulation of a nondialyzable ribosomal breakdown product which has chromatographic (MAK) properties similar to t-RNA. Further evidence that this may be a ribosomal breakdown product was obtained by a base ratio

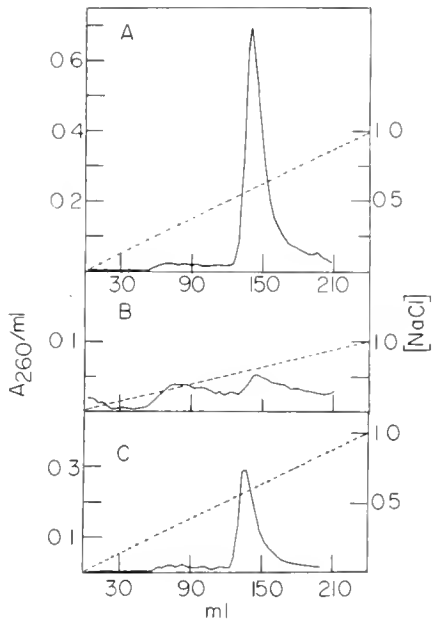

Fig. 16.

The fractionation of total soluble RNA from epithelial cells and fiber cells on DEAE-cellulose. (A) Total soluble RNA from calf cortex fiber cells. The ribosomal RNA and soluble RNA were first separated by sucrose gradient. The soluble RNA fractions from the top of the gradient were combined and dialyzed against 0.01 *M* Tris-HCl pH 7.3 + 0.01 *M* $MgCl_2$ +5 μg/ml PVS before being placed on the column. (B) Total soluble RNA from calf epithelial cells. The experimental procedures were the same as in A. (C) Total soluble RNA from calf cortex fiber cells. The ribosomal RNA and soluble RNA were first separated by fractionation on a MAK column. The soluble RNA (peak A) fractions were combined and dialyzed as described in Fig. 16A before being placed on the column. (Fig. 14, J. Papaconstantinou, *Science*, In press; copyright by the American Association for the Advancement of Science.)

analysis of the ribosomal RNA eluted from the DEAE column. This fraction has a GC content of 64%, which is typical of ribosomal RNA. As a further characterization of these fractions we are presently studying the extent to which they can be charged with C^{14}-amino acids.

C. Inactivation of DNA: the loss of nuclear activity in lens fiber cell

The MAK column patterns in Fig. 14 indicate that there is a significant decrease in the DNA in the cortex fiber cells. These observations are in agreement with cytological reports that the nucleus of the cortex fiber cell decreases in size and is ultimately lost in

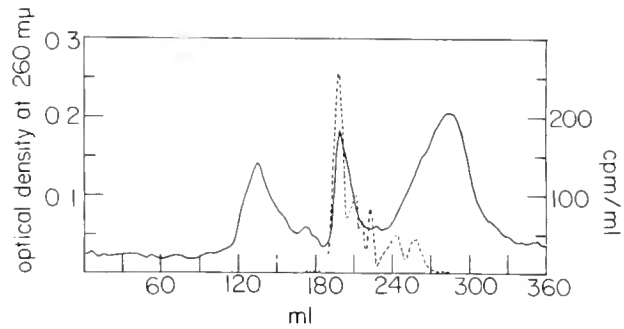

Fig. 17.

The fractionation of phenol-extracted nucleic acids from calf lens epithelial cells incubated in ^{3}H-thymidine. O.D. $_{260}$, ———; counts per minute/ml -------. (Fig. 15, J. Papaconstantinou, *Science*, in press; copyright by the American Association for the Advancement of Science.

the older fiber cells.

As I stated previously, the lens fiber cells do not have the ability to replicate. Although small amounts of DNA could be detected in the nucleic acids extracted from the fiber cells it is not known whether this DNA is metabolically active. In view of this, we performed experiments to determine whether ^{3}H-thymidine is incorporated into the DNA of the fiber cells. After incubating calf lenses in ^{3}H-thymidine, the nucleic acids from epithelial cells and fiber cells were phenol extracted and fractionated on MAK columns (Fig. 17). The DNA (peak B) fractions were counted and it was found that the ^{3}H-thymidine was incorporated into the DNA of the epithelial cells. On the other hand, the corresponding experiment with the fiber cell DNA fraction showed that there is no incorporation of ^{3}H-thymidine into the fiber cell DNA. We concluded from these experiments that although the DNA of the epithelial cells is metabolically active, this activity is lost in the fiber cell. This observation, we feel, has an important and obvious bearing on our observations that the m-RNA in the fiber cell is stable.

V. Summary and Conclusions

I would like to return to the beginning of my lecture, where I listed the cytological events marking the differentiation of the lens epithelial cell to the fiber cell and now attempt to correlate these events with our biochemical observations. The morphological and biochemical alterations characteristic of all stages of lens cell

differentiation are shown in Fig. 2. Firstly, the epithelial cells have basophilic staining properties whereas the fiber cell has acidophilic staining properties. This change in staining properties may have been brought about by the synthesis of γ-crystallins. These proteins are slightly basic having isoelectric points ranging from pH 7.5 - 9.0. In view of their basic properties the γ-crystallins may bind to the nucleic acids of the nucleus and cytoplasm thus effecting the observed alteration in staining properties. It should also be pointed out that the isoelectric points of the α-crystallins is 5.2 and of the β-crystallins ranges from 6.0 to 7.0. Both these groups of proteins as well as the rich population of ribosomes would contribute to the basophilic staining properties of this cell.

In addition to the change in staining properties there is also a conversion from a rough endoplasmic reticulum in the epithelial cell to a smooth endoplasmic reticulum in the fiber cell. The breakdown and subsequent decrease in the ribosomes may be directly related to the appearance of the endoplasmic reticulum. At present I cannot present any information on the mechanism of this ribosomal breakdown. The fact, however, that there is also an overall decrease in the rate of protein synthesis in the fiber cells may be a consequence of the ribosomal breakdown.

The increase in the size of the nucleus and nucleoli and the increase in the ribosomal population at the time of cellular elongation might indicate the initiation of an overall synthesis of materials required for the morphological changes of the cell. Although we know nothing of the function of γ-crystallins, it has been shown that the synthesis of this major group of proteins is initiated during cellular elongation. In addition to the increase in protein synthesis there must also be an increase in the synthesis of nucleic acids, both ribosomal and m-RNA. The synthesis of these two classes of RNA may account, therefore, for the morphological changes in the nuclei and nucleoli.

In the fiber cell it has been observed that there is a gradual decrease in the size of the nucleus and as the cell gets older the nucleus disappears. Through the use of ^{3}H-thymidine we have shown, as would be expected in a replicative cell, that the epithelial cells contain metabolically active DNA whereas the fiber cells no longer have the capacity to incorporate precursors into its DNA. Furthermore, the data from our MAK columns have shown that there is a significant loss of DNA in the fiber cell. Both of these observations are in complete agreement with the cytological observations on the fate of the nucleus in lens fiber cell differentiation.

The loss of nuclear activity brings up the question of the synthesis of m-RNA for the continuation of protein synthesis. Upon inactivation of the nucleus, the synthesis of m-RNA stops and the cell would require some mechanism for the conservation of existing m-RNA for the continuation of protein synthesis. The stabilization of m-RNA in these fiber cells has been shown; the mechanism of stabilization, which has not been worked out, should prove to be an important one for understanding the molecular aspects of terminal cellular differentiation.

I have presented just a limited spectrum of the macromolecular interactions which occur during the terminal stages of lens cell differentiation. There are many cell types which undergo similar morphological alterations during their terminal stages of differentiation. These cells are also involved in the synthesis of highly specific proteins such as hemoglobin, myosin and keratin and there are indications that the same molecular alterations described for the lens may also occur in these cells. Thus, a specific series of macromolecular interactions such as those described above may be a basis for the biochemical definition of the terminal stages of cellular differentiation. The differentiation of the reticulocyte, for example, involves inactivation of the nucleus and stabilization of m-RNA. It remains to be seen whether there also occurs a ribosomal breakdown and the accumulation of a breakdown product such as I have described here. Furthermore, the elucidation of the mechanisms of reactions involving nuclear inactivation, the stabilization of m-RNA and the breakdown of the ribosomes may be the basis of the mechanisms of terminal cellular differentiation. This is important because most cells exhibiting the property of synthesizing highly tissue specific proteins, enter terminal stages of differentiation and exhibit molecular properties similar to those described above.

The lens cell has reached its highest form of cellular differentiation when it has formed the fiber cell, and as it approaches this stage it develops very specific metabolic activities. With respect to the mechanism of lens fiber cell formation, therefore, one would ask how much of this metabolic activity is dependent upon the morphological changes and whether

these biochemical interactions are intimately associated with the genetic regulation of morphogenesis. To be more specific, we would like to know whether γ-crystallin synthesis is intimately linked to fiber cell formation and whether the γ-crystallins are required to bring about the formation of a fiber cell. The potential for synthesizing γ-crystallins is inherent in the genome of the cell. This part of the genome is non-functional in the epithelial cell. Can these genes be activated without bringing about a simultaneous (a) cellular elongation; (b) loss of cellular replication (c) stabilization of m-RNA and (d) breakdown of the ribosomes? The degree of coupling or uncoupling of tissue specific protein synthesis to morphogenesis is an important part of the mechanism of cellular differentiation. We feel that we have now reached the stage where we can begin to answer these questions.

MASSARO: I have a couple of questions if you wouldn't mind going back to LDH. What other data do you have besides the pyruvate inhibition curves to show that the LDH-1 of your lens system is different from the LDH-1 of the skeletal muscle, heart, brain, glands system?

PAPACONSTANTINOU: Well, we don't have any other evidence.

MASSARO: Recently we've found that in fish the LDH system of the eye apparently differs from the skeletal muscle, heart, glands and CNS system. I would be kind of shaky about making a very strong statement concerning the results Cahn got in tissue culture and the results one obtains with aging muscles because we just don't know much about what goes on during the aging process. Also, there are dedifferentiation and other problems in tissue culture.

GROSS: Did you say that the behavior of your LDH-1 with respect to pyruvate was different from muscle LDH?

PAPACONSTANTINOU: No, it's the same. We have no evidence that they're different at all.

MASSARO: Doesn't your LDH-1 differ from the LDH-1 of the muscle tissue?

PAPACONSTANTINOU: No, it's the same; it's sensitive to pyruvate. The difference is that the fiber cells retain LDH-1 although they have a high rate of aerobic glycolysis, and they should have LDH-5.

MASSARO: Are all of the properties of this LDH-1 similar to the LDH-1 of the muscle?

PAPACONSTANTINOU: They are similar to those of the heart muscle.

MASSARO: The only difference is that in this particular environment you have an LDH-1 which is sensitive to a clearly known pyruvate concentration?

PAPACONSTANTINOU: That's right. What we're trying to point out here is that the fiber cells are highly glycolytic and according to the theory that's been proposed for heart and skeletal muscle, the fiber cells should retain LDH-5; instead they retain LDH-1.

MASSARO: Then, the only difference that you see here is in the pyruvate inhibition curves?

GROSS: The proposal about LDH and the oxidative level is, as I understand it, not a theory, but an explanation of why you have more of one enzyme in one tissue than another. You are suggesting that differential gene action results from the influence of the environment of the cell. In the classical cases, the influence was pinpointed as oxygen tension or as the state of carbohydrate metabolism. Now, as I understand it, Papaconstantinou's evidence showed that, in his system, this explanation is invalid. My conclusion from that would be that the initial explanation is not universally applicable.

MASSARO: This conclusion is on the basis of the pyruvate inhibition?

GROSS: Yes.

PAPACONSTANTINOU: It's based on the fact that LDH-1 persists in a highly glycolyzing system that can go into oxygen debt.

MASSARO: You see, I think we're running into some semantic problems here by labeling this LDH-1.

PAPACONSTANTINOU: Well, I can't call it heart-type LDH because it's not in the heart.

MASSARO: We're crossing over systems and using the LDH terminology of the heart and skeletal system in the eye system. I don't think this is valid because I don't think they're comparable. I think, perhaps, from this data that the eye LDH system may be quite different from that of skeletal muscle and heart muscle.

GROSS: What you're saying, then, is that there are, in fact, different cistrons involved.

MASSARO: That's right.

GROSS: The initial assumption in this story was that there are two genes involved and they're the same in every genome. Now, you're suggesting, in fact, that there are other genes involved.

MASSARO: We know this for a fact in the case of the so-called "X-bands of gonadal LDH"

PAPACONSTANTINOU: I would not expect to find differences in amino acid composition between lens LDH and heart LDH from the same animal. Genetically the two LDH's should be

the same. I think, however, that the point I am trying to make with respect to the regulatory mechanism has nothing to do with the genes; it's at an entirely different level. The only genetic involvement in this work is the regulation of subunit synthesis.

MASSARO: On the basis of the pyruvate inhibition curve alone, I don't think you can emphasize that these LDH's are one and the same.

GROSS: It seems to me if you have two different genes, then the original explanation is wrong on the basis of Papaconstantinou's results. If you don't have two genes, then this is another complicated case of differential gene action and not very much more can be said about it.

HYMER: Have you studied the process of ribosomal breakdown during differentiation to the fiber cell by electron microscopy?

PAPACONSTANTINOU: With electron microscopy there is a decrease in the ribosomal population. In other words, if you count the ribosomes in a unit area, you'd find a decrease in the actual number of ribosomes.

HYMER: Are these membrane bound?

PAPACONSTANTINOU: No, they're not membrane bound, especially in the fiber cells.

KOHNE: The one criticism that you could make of this is that RNA breakdown is something other than ribosomal breakdown since all you've got is phenolyzed RNA.

PAPACONSTANTINOU: Well, there is no ribonuclease in the lens system, that we can detect. I apologize to everybody who has ribonuclease troubles, but we just don't have them. We used 5 gammas/ml of polyvinylsulfate in our phenol extractions but it doesn't make any difference whether we use it or not.

POLLARD: How does ribosomal RNA break down?

PAPACONSTANTINOU: We don't know yet. It's been a very interesting phenomenon. If ribonuclease is present we ought to get breakdown products smaller than what we observe, unless it's a special ribonuclease, which I'd like very much to find out.

GROSS: You remember Nemer's paper on RNA synthesis during the early development of the sea urchin, in which he showed gradients with RNA's that looked like 28s, 18s, 13s, 10-13s and 4s? The 28s RNA in animal cells of this sort does, in fact, break down into things that sediment roughly at 10-13s.

PAPACONSTANTINOU: Because of the high GC content of the breakdown product I am inclined to believe that it comes from the 50s ribosomal particle. This is the part of the ribosome that 28s RNA is derived from. However, this is just speculation right now. We are trying to characterize the 50s and 30s ribosomal RNA to determine if the breakdown product originates specifically from either one or both.

EPEL: Can you say a little more about the temporal relationship between the ribosomal breakdown and the lens protein synthesis?

PAPACONSTANTINOU: Although we don't really have kinetics or good turnover data, indications are that lens protein synthesis decreases about 10-fold in going from an epithelial cell to a fiber cell. Paul, did you mention the fact that there was a protein that was involved in the stabilization of messenger RNA in the early embryo?

GROSS: It's an idea that's been suggested. Monroy and his colleagues found that trypsin treatment of unfertilized ribosomes allowed them to work.

PAPACONSTANTINOU: If we prepare ribosomes from epithelial cells and fiber cells and do an RNA-protein ratio, the RNA-protein ratio in the epithelial cells is about 0.5 to 0.8 (they're good ribosomes). When you do it in the fiber cell, it goes down to 0.1 and sometimes less. There's an indication that there's protein being stuck onto the ribosomes of the fiber cells which may explain that smooth endoplasmic reticulum and possibly the stabilization of messenger.

GROSS: Couldn't there also be isolation artifacts since you're making lots of the crystallins and what not?

PAPACONSTANTINOU: Well, no. We're extracting under the same conditions. Why shouldn't we get a lot of protein on these ribosomes then?

GROSS: Because it's a different kind of protein.

PAPACONSTANTINOU: The only difference we have is the formation of the gamma crystallin. The gamma crystallin is a basic protein relative to the others. The gamma crystallins could be sticking onto those ribosomes. They may be a stabilizing factor. This is a highly specific protein that is formed in fiber cell formation. This is a real speculation, though. It's a highly specific protein that's associated mainly with fiber cells and it comes up just before the stabilization of the messenger. I don't know why they would want so much gamma crystallin for the stabilization.

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PROLIFERATION AND DIFFERENTIATION OF STEM CELLS OF THE BLOOD-FORMING SYSTEM OF THE MOUSE

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First, I'll describe the system with which we've been working, and, then, in the remaining time, I'll tell you something of what we've been doing with it. The work I'll describe was done in collaboration with Dr. Ernest A. McCulloch and Dr. Louis Siminovitch.

The method we use to detect and count stem cells has been described in detail elsewhere (1, 2). The method is based on the transplantation of cells into heavily irradiated recipient animals. The irradiation converts the recipients into culture vessels in which the transplanted cells can grow by destroying the proliferative capacity of the animals' own cells. Inbred strains are used to avoid transplantation difficulties. One can regard this as a form of cell culture *in vivo*. The irradiated recipient is well designed for this purpose, since it has a built-in temperature control, a built-in pH control and a built-in medium supply.

Cells are taken from a normal donor, suspended, counted and injected intravenously into the irradiated recipients. Cells from any blood-forming tissue may be used; we usually use marrow. If you wait 10 days and look at the spleens of these animals, you find colonies in their spleens. These are colonies of cells that have grown up from those cells of the transplant which lodged in the spleens of the irradiated animals. When the colonies are fixed in Bouin's solution, they turn yellow, and you can count them very easily. The number you get is proportional to the number of cells you put in. We find about 10 colonies per spleen per 10^5 cells injected. Why is there this rather low efficiency? We think it is because most of the cells that go into the mouse are fully differentiated, or almost fully differentiated; that is, they are cells that don't have much proliferative capacity left. Certainly, they do not have enough to make a colony of this size which contains something like a million cells.

POLLARD: That's not straight dilution, is it?

TILL: No, we've measured that. About a fifth go to the spleen, so it's not that only a small number get there (3). The spleen is a pretty efficient filter of cells put into the blood stream.

The other point I want to make is that the relationship between the number of colonies formed and the number of cells injected is linear and extrapolates back through the origin. This suggests that the colonies are formed by single entities which lodge in the spleen (1, 2).

POLLARD: Have you done the Poisson test on this?

TILL: Yes. The distribution of the number of colonies per spleen, for colonies formed by transplanted cells, appears to be a Poisson distribution.

If you look at the colonies using histological methods, you find that they contain differentiated cells. Thus, these colonies are not like the colonies that are formed by bacteria, for example, where the cell composition is fairly uniform. The cell composition of these colonies is heterogeneous; they contain differentiated cells and often more than one kind of differentiated cell. These differentiated cells are blood cells or their precursors, that is, erythroblasts, granulocytes and megakaryocytes. So this raises the question, are these colonies formed by the differentiation of more than one initiating cell or does this mixture arise from a single precursor? It's a rather important question, so we have tried to find out about this.

Dr. Andrew Becker did these experiments (4). What he did was to obtain spleen colonies from irradiated marrow cells. The irradiation

inactivates some of the colony-forming ability of these cells, so he had to start with more cells (about 100 times as many) in order to end up with discrete colonies. Then individual colonies were picked out of the unfixed spleen. The individual colonies were separately dispersed and chromosome preparations were made and examined.

At the dose that was used (650 rads) about 10% of the colonies contained chromosome aberrations. The point of interest was that whenever a chromosomal aberration was found in a dividing cell in a colony, the same aberration was present in more than 95% of the other dividing cells in that colony. The interpretation was that the irradiation of a single precursor had generated the chromosomal aberration which was then passed on to all its descendants, and that, in fact, the colony was a clone formed from this original damaged precursor. With ionizing radiation, aberrations are formed in a random fashion, so each aberration that you get is different. He found a total of 8 marked colonies. Each one had a different kind of aberration, but all appeared to be clones because all the dividing cells of each colony carried the marker characteristic of that colony.

This doesn't really settle the question of whether the different kinds of differentiated cells have come from a common precursor because here we have just looked at dividing cells. On the other hand, most of the differentiated cells no longer are dividing. We haven't conclusively proven that the different kinds of differentiated cells we find in a single colony have arisen from a single precursor but this is fairly good indirect evidence.

We've taken as a working hypothesis that the spleen colonies are clones, and that these clones are formed by some sort of precursor cell which can differentiate in multiple ways. What one would like to know is, what governs that choice? What determines whether the cell will give rise to erythrocytic precursors or granulocytic precursors or megakaryocytes? This is what we're primarily interested in.

Perhaps I should just refresh your memories for a moment about the organization of a renewal system like this. It's been postulated for a long time that there exist stem cells which have two functions: one is to maintain their own numbers; the other is to begin the pathway of differentiation so one gets a series of divisions resulting in cells with different functional characteristics, thus ending up with a wholly differentiated, fully specialized cell such as the red blood cell. This latter cell can't divide; it hasn't even got a nucleus. Its immediate precursors can divide a few times, but capacity for proliferation is limited and they have begun to differentiate and form hemoglobin. The ones nearest the stem cell can divide a lot and contain no hemoglobin. The fully differentiated cells are continually lost from the system, and they must be replaced somehow. Since they can't divide themselves, they must be replaced by the division of the precursor cells. The ultimate cell, the cell that has no precursors after the embryonic stages, is the stem cell. Although this type of cell was postulated to exist, it proved difficult to obtain clearcut ways of recognizing it experimentally. The spleen-colony technique appears to be one way of doing it. This method makes use of the major function of the stem cells, which is to proliferate, and demands that they be able to multiply through a number of cell generations sufficient to give rise to a colony of cells that you can see with the naked eye. It's an arbitrary criterion, so we probably don't detect all the stem cells by this criterion; but we do see some. It gives us a class of stem cells that we can look at.

If this is true, we would like to know what regulates the proliferation and differentiation patterns of the stem cells. The stem cell, if it is really multipotent, has several choices open to it. One can postulate four of these. First, it may choose to proliferate so as to maintain its own numbers. We could call this, for the lack of a better term, "self-renewal," although there is evidence that the daughter stem cells may not be exactly like their parents (5). If the system is to keep going, the stem cells need to be able to perform some sort of self-renewal, because, by definition, there is no precursor for them in the adult animal. Second, the stem cell may differentiate to give rise to cells of the red cell series. Third, it may give rise to cells of the granulocyte series. Fourth, it may give rise to megakaryocytes. Thus, four different pathways of proliferation or differentiation are available to the stem cell. One would like to find out what governs whether or not a particular choice is made.

How can one go about trying to solve this problem? Professor Pollard suggested this morning that one way of approaching a problem of this type is by the use of genetic methods, and this is what we have tried to do. If you can find a single gene mutation that affects some step in the process, then you can assume that the molecular basis for that particular effect is

probably relatively simple. If it were not, it wouldn't be under the control of a single gene. In the mouse, there are several mutations which are known to produce anemia, *i.e.*, to affect in some way one of the pathways of differentiation in which we are interested. Some of these have already been studied in considerable detail by Dr. Elizabeth Russell and Dr. Seldon Bernstein at The Jackson Laboratory, Bar Harbor, Maine (6). The question is, how do these genes affect the pathways of differentiation of the stem cell?

Let's imagine what kinds of genetic changes one could see, speaking of gross changes rather than individual steps. Each one of the four suggested pathways open to the stem cell, presumably, could be regulated by a separate gene. There might be a gene that regulates self-renewal. For example, there may be some molecule that triggers off the self-renewal division, since there is evidence that it isn't happening all the time (7). Most of the stem cells are not dividing and one may postulate that some of them are triggered off by a gene product. You can imagine four classes of these genes corresponding to these four possible pathways that this stem cell can go into. You can imagine, also, two general ways that regulation could occur. It could be a property intrinsic in the stem cell itself which regulates it or the regulator could come from outside. Let me just take an example; let's say that regulation might depend on the permeability of the membrane of the cell. That would be something I would call intrinsic to the cell itself. On the other hand, the regulator could be a hormone that comes in from outside and tells the cell to do something. That I would call external because the mutation would then be a mutation that stops the supply of that hormone, whereas the mutation in the first case would be one that alters the membrane. So there are two classes, intrinsic and extrinsic, each of which can be applied to each of the four suggested pathways.

We have looked at three mutations in some detail so far; and we think that each falls into a different class. One of these is extrinsic in its action and the other two are intrinsic. I'll describe the evidence for this next.

The mutations we've looked at have been *W*, *Sl* and *f*. Let's consider *W* and *Sl* first. The animals that we studied were of genotype W/W^v and Sl/Sl^d, obtained from Drs. Russell and Bernstein. Animals of these two genotypes have very similar phenotypes. Both have a severe macrocytic anemia, the coat color is affected, the animals are sterile and they are very radiation-sensitive. Superficially, the phenotypes appear almost identical. However, we know they're different because they map at different genetic sites. If the cells that form colonies in the spleen are a kind of stem cell and the basis for these anemias is a defect in the stem cell, then we should find deficient colony formation when we test the cells from these animals for their ability to form colonies in irradiated normal hosts.

For W/W^v we found deficient colony formation (8), and the subsequent work that's been done indicates that this may be a defect in the ability of the stem cell - the colony-forming cell - to renew itself. Therefore, this is a mutation whose effects are intrinsic to the stem cell. It's apparently a defect at the cellular level in the ability of the cell to produce more cells like itself. Because the phenotype was similar, we expected that the stem cells in animals of genotype Sl/Sl^d would be similar in their properties to those of animals of genotype W/W^v. In fact, they turned out to be very different. When we tested cells from Sl/Sl^d mice for their ability to form colonies in our standard test system (irradiated normal mice), we found that they formed colonies perfectly well (9). Every attempt that we made to find a defect in the composition of these colonies failed; they are apparently perfectly normal. So then we did some experiments the other way around. We used these animals as recipients for normal cells. We had found previously that W/W^v animals are good hosts for the growth of normal cells (8). You can put normal stem cells into them and they grow well. In fact, you don't even have to irradiate the hosts first. You can put normal cells into unirradiated mice of this genotype and they'll still form spleen colonies. In fact, they'll cure the anemia of the animal permanently (10). Thus, animals of genotype W/W^v exhibit a defect at the cellular level, but the host is capable of supporting the growth of normal blood-forming cells. However, if you put normal cells into Sl/Sl^d mice, they don't form colonies. Even if you irradiate these animals with large doses, they won't support detectable growth of normal cells (9). In this case, the cells seem to be normal, but the mouse does not support their growth normally. Thus, this mutation affects a process which is extrinsic to the stem cells.

One would expect, if all this is right, that the cells from Sl/Sl^d would grow in W/W^v hosts. In other words, one should be able to take cells from an anemic animal of genotype

S1/S1^{d}, put them into an anemic animal of genotype *W/W*^{v} and cure the anemia. This experiment has been done by Russell and Bernstein, with whom we collaborated in these experiments, and it worked perfectly well. The animals are cured of their anemia at least as well with cells from *S1/S1*^{d} animals as with genetically normal cells (9). This is complementation of the cellular level.

KAHN: Where did the stem cells come from in the *S1* type? If you have no stem cell renewal, how do you get the spleen to pick up cells?

TILL: We don't know the answer to that. Maybe this is not as serious a defect in the embryo and enough multiplication of the stem cells can occur. Also, we can't rule out that there's slow multiplication. Perhaps over a long period of time these cells can build up in numbers. We don't know yet which is right. Our experiments on growth in these hosts have been carried out over a fairly limited period of time - about two weeks. I think the fact that these animals are alive and that they do have normal numbers of stem cells in them means that there must be slow growth of stem cells until the equilibrium level is reached. However, the evidence does suggest that the *W* gene acts intrinsically and the *S1* gene extrinsically to the stem cells.

In the *S1/S1*^{d} animals one would like to know, what is the external regulation? Is it by means of a molecule? Can the molecule be isolated? We did standard experiments to test for such a molecule. An *S1/S1*^{d} animal was joined in parabiosis with a normal animal of the same inbred strain; that is, they had a shared circulation. We demonstrated the existence of the shared circulation by putting chromium-labeled erythrocytes into one animal and showing that they appeared in the other. Then both were irradiated and inoculated with the same number of normal bone marrow cells. If the failure of cells to grow in the *S1/S1*^{d} irradiated host is due to an inhibitor which is circulating in the peripheral blood, then we should get growth in neither member of the parabiotic pair. If, on the other hand, it's the lack of a stimulatory factor that accounts for the failure of cells to grow in *S1/S1*^{d} hosts, in the parabiotic situation this factor should be supplied by the normal member of the pair and there should be growth in both. In fact, what we got was exactly the same situation as if they hadn't been joined together (9). In other words, the cells grew in the normal animal and they didn't grow in the mouse of genotype *S1/S1*^{d}. Either there is not a factor that circulates in the peripheral blood or it's so unstable that before it can get from the normal to the anemic mouse it's gone. We don't know which it is, but this just makes the whole system difficult to study, because it means one is looking at relatively short range factors. We haven't yet had any good ideas on how to investigate a short range factor of this type.

The third type of mutant that we have studied, *f/f* has quite a different kind of anemia. It's a transitory anemia which just shows up in the embryo and the new born and apparently cures by the time the animal is two weeks of age (11). The first experiment we did on mice of genotype *f/f* was as follows: we thought a transitory anemia might be more interesting because we might see shifts in the properties of the cell depending on the age of the animal we took them from, so we took animals of this genotype of various ages and tested their marrow cells for ability to form colonies, as compared with cells from normal animals of the same inbred strain. We found that marrow cells from the controls and from the mutant animals produced spleen colonies with the same efficiency. In other words, we got the same number of colonies from the same number of marrow cells transplanted. However, in the case of cells from *f/f* donors, independent of their age, the colonies were different in composition. They were smaller, and when we tested them in various ways, they showed fewer erythrocyte precursors. Experiments done in collaboration with Dr. Margaret W. Thompson and Dr. John Fowler (12, 13), have indicated that this deficiency is specific, in that both the number of granulocytes and the number of new stem cells, produced by rapidly proliferating transplants derived from *f/f* donors, appears to be normal. Apparently, the defect in cells from mice of genotype *f/f* causes them to be late in arriving at the stage where they begin hemoglobin synthesis. If the cells are stimulated to proliferate at their maximum rate, then this defect appears in cells from *f/f* donors, independent of their age. Thus, the "curing" of the anemia in adult mice is not due to repair of the defect, but is apparently, the result of its being masked by the decreased demand for production of new erythrocytes in the adult animal.

In any event, the defect in *f/f* mice appears to be intrinsic to the stem cells, and seems to affect their ability to differentiate toward the production of red cells. What the exact nature of the defect is, we don't know. Of course, there is a hormone which specifically stimulates cells of the blood-forming system to proliferate and

to produce red cells. That is the hormone erythropoietin (14). It's possible that this mutation is affecting the cells that have committed themselves to erythropoietic differentiation but have not actually begun to synthesize hemoglobin, and that what it does is to decrease the ability of these cells to respond to erythropoietin. This is one possible hypothesis - that they do not respond normally to erythropoietin, so they are late in initiating hemoglobin synthesis.

Now, if this view is correct, the defect in mice of genotype *f/f* is manifested at a stage prior to the initiation of hemoglobin synthesis. Apparently, here is a case of a cell being already committed to erythropoietic differentiation, since the defect appears to be specific for that pathway; and yet the defect is manifested at a stage prior to the synthesis of hemoglobin. It seems that there must be a whole set of controls of early differentiation which act well before the cell actually begins to differentiate to synthesize hemoglobin. Thus, these controls may have nothing to do with regulation of the stable messengers or ribosomes involved in hemoglobin synthesis, since they're acting at stages much earlier than that. One would like to know whether or not this type of early differentiation involves a different means of regulation.

GROSS: Why do you think that kind of regulatory process would involve things other than ribosomes and messenger and what's your suggestion for the other things?

TILL: Simple prejudice. I have no evidence whatsoever.

GROSS: I've heard other people make that statement. It would be very nice if one knew about these things. However if you want to make hypotheses that one can test about what makes a cell decide to do something, you have to use things that you know about.

TILL: Let me take a possible example. There is a system in the mouse which regulates whether or not a transplant will grow, if you take it from one inbred strain to another. It's the histocompatibility system. There are several genes that have been mapped that are concerned with this. The principal one is the H-2 locus. This is a complex locus but apparently one of its functions is to control the synthesis of an antigen on the surface of the cell. This antigen is what the new host reacts to if you transplant the cells into an unrelated host. Since such transplantations of cells do not occur in nature, one may ask: What is the normal function of the surface antigen, if any? One suggestion which has been made is that the real function of the H-2 locus is a regulatory function in the normal animal (15). If so, the antigen we detect when we transplant cells from one animal to another is part of a normal regulatory system. This antigen is known to be on the cell surface and it's possible that cell surfaces are what are involved in regulation of this level.

GROSS: I'm still afraid that it is just a matter of principle which is under discussion. It's entirely conceivable that cell surfaces do differ dramatically from one differentiation path to another, and that if they do differ, they differ because their macromolecular content is different. If they have different proteins, then the initial event still has to arise in the genome.

TILL: I think there is a misunderstanding here. I'm certainly not suggesting that all the recent work on the mechanisms of regulation of protein synthesis is incorrect, nor that there is some new system of protein synthesis that has nothing to do with messengers or ribosomes. It is possible that the initial stages in the regulation of the proliferation and differentiation of erythropoietic cells may have nothing directly to do with the production of messenger for the synthesis of hemoglobin. The synthesis of hemoglobin may represent a very late stage in the differentiation process. If so, then perhaps studies of the control of the formation of messenger for the synthesis of hemoglobin by cells may tell one relatively little about the very early steps of differentiation. The initiation of hemglobin synthesis may be just one byproduct of other events which took place long before, in the history of the progenitors of those cells.

POLLARD: Have you done any fractionation at all of the cells at this stage where erythropoietin might take over?

TILL: No. A number of people are doing this, though.

POLLARD: If it's the surface that you're talking about, then this is a place where a lot of material is located.

TILL: Oh yes, I think one could get at this experimentally.

EPEL: Can you transfer these cells to other parts of the body? Do they go any other place and proliferate?

TILL: Yes, the cells that we put into irradiated recipients seem to proliferate in any place where hemopoietic cells should proliferate.

PAPACONSTANTINOU: Do these others show the same composition that you get in the spleen?

TILL: Yes. They're just easier to see in the spleen. Fetal liver, which is a blood forming tissue, also will make spleen colonies which look very much like the colonies you get from adult marrow.

This brings up another point about fetal and adult hemoglobin. I was quite interested in your suggestion that you get a different number of your LDH set predominating, depending on whether the cells are proliferating or not, because this same thing has been suggested for adult and fetal hemoglobin (16). It's not the adult versus the fetus so much that counts; it's how fast the cells are multiplying. This is hard to test but it has been suggested.

PAPACONSTANTINOU: We might be able to use our system for such a test if we could place fetal cells in culture and get them to differentiate.

TILL: I'm hoping we'll be able to test this for fetal versus adult hemoglobin.

GRUN: There's a possibility that you could get at this question of inhibition versus the possibilities of supplying the missing ingredients by crossing these two mice. Then, if there were an inhibitory substance produced by one mouse, you might expect the hybrid, also, to have this inhibitory process produced by one of the alleles.

TILL: The trouble with the hybrid (containing two mutant *S1* and two mutant *W* alleles) is that it's got both things wrong with it. It's got a defect in the cell *and* a defect in the host. So it's not the same as doing a transplant.

J. WRIGHT: Have you used hybrids?

TILL: Hybrids have been made at The Jackson Laboratory, and I believe that they die right away.

GRUN: I suppose you could try to go at it by crossing the mutant with the normal.

TILL: The cross is a different thing. The transplant lets you vary the genetic composition of the cell independently of the genetic composition of the host, which you cannot do by any kind of a cross that you can devise.

KOHNE: Can the mosaics of these mice live?

TILL: That I don't know about. We've essentially made a mosaic but I don't know of a naturally occurring one. I gather it is technically feasible in a developing system at a very early stage, after fertilization, to make a composite embryo and get a mosaic that way (17). However, I don't think it's been done with these mutants.

KOHNE: How long do the chimera live?

TILL: Dr. Beatrice Mintz has raised them, but she works primarily with a *t* mutation. I don't think she's tried this with these mutants.

EPEL: Are the kinetics of the iron uptake in the *f/f* cells the same as the controls once it starts?

TILL: The uptake per synthesizing cell appears to be the same in rapidly proliferating cells from *f/f* animals as in cells from controls. The proportion of cells which are undergoing synthesis is less for the cells from *f/f* mice than in the controls (13).

GRUN: Maybe I'm missing a point in this thing. The question that you're asking is, does this *S1/S1*^{d} mouse form something which is inhibitory in the developing animal or not? Now, if you formed an *S1s1* the *S1* allele in the heterozygote would presumably still be forming inhibitory substance if there is an inhibitory substance there and the *S1* is a dominant condition.

TILL: It isn't. One sees a very nearly normal blood picture. Now, this suggests to me that *S1* is failing to supply some nutritive requirement to the stem cell rather than that *S1* is forming an inhibitory substance.

GRUN: In that case, in the parabiotic it should have been filled.

TILL: It might still be unstable. We were very disappointed in the parabiosis experiment.

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THE STRUCTURE OF ISOZYME SYSTEMS AND THEIR ROLE IN DEVELOPMENT

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In recent years, the study of isozymes has expanded to such a degree that an attempt to cover the field at a conference of this nature would probably be only of minimal value. Therefore, what I intend to do today is to use the lactate dehydrogenase system of isozymes as a model and present to you a fairly detailed view of some of the work that has been and is being pursued in this area.

The individuality of cells, that is their phenotype, is expressed in large measure by the activities of their constituent enzymes. These enzymes are the products of a complex series of metabolic events that are under genetic control. In the broadest terms, enzyme biosynthesis involves DNA transcription into RNA and RNA translation into the linear amino acid sequence or primary structure of a polypeptide chain. Each polypeptide chain then assumes the characteristic three dimensional conformation of its secondary and tertiary structure. In numerous instances these intricately folded polypeptide chains are enzymatically inactive until they become aggregated into more complex units (the quarternary structure of the enzyme).

The essence of the relationship between DNA and enzyme structure has been summed-up in the so-called one gene-one enzyme hypothesis. A logical consequence of this hypothesis is that the cells of a homozygous organism should synthesize identical replicas of all of their constituent protein molecules. But it has become abundantly evident in recent years that numerous proteins, including many enzymes, exist in several physically distinct forms within the cells of a single organism. The multiple molecular forms of enxymes have been termed "isozymes" (1).

The isozymes of LDH exhibit both species- and tissue-specific patterns (2). Furthermore, during the course of embryonic development, these patterns undergo profound, albeit gradual, changes (3). From such observations it seems reasonable to propose that the remarkably characteristic isozyme pattern of each tissue reflects a physiological uniqueness of the individual isozymes which is superimposed upon their essential similarities. The existence of isozymes then poses important questions concerning their biosynthesis and their enzymatic and physiological activities.

LDH is ubiquitously distributed in isozymatic forms among vertebrates and also occurs as such in numerous other organisms. It is an oxido-reductase catalyzing the interconversion of lactate to pyruvate. This reaction is mediated through the cofactor nicotinamide adenine dinucleotide (NAD). During periods of relative anaerobiosis the enzyme functions to provide a reservoir for the storage of hydrogen by forming lactate which occupies a metabolic dead end. This aids in maintaining the supply of NAD needed at an earlier step in the glycolytic pathway. When adequate supplies of NAD are again available, lactate is oxidized to pyruvate. These reactions are summarized in Fig. 1. It should be noted that all the enzymes indicated on this chart, except the three denoted by an asterisk, have been shown to exist in multiple molecular forms. Recent evidence indicates that triosephosphate isomerase also probably exists in isozymic forms.

Although early investigations had undoubtedly demonstrated the existence of multiple molecular forms of LDH, their biological significance was not at first recognized (4, 5). In 1957 Vesell and Bearn (6) described the existence of three LDH isozymes in human serum and changes in their proportions during various disease states. About the same time, Wieland and Pfleiderer (7) independently discovered the existence of multiple molecular forms of LDH and demonstrated tissue specific patterns of the

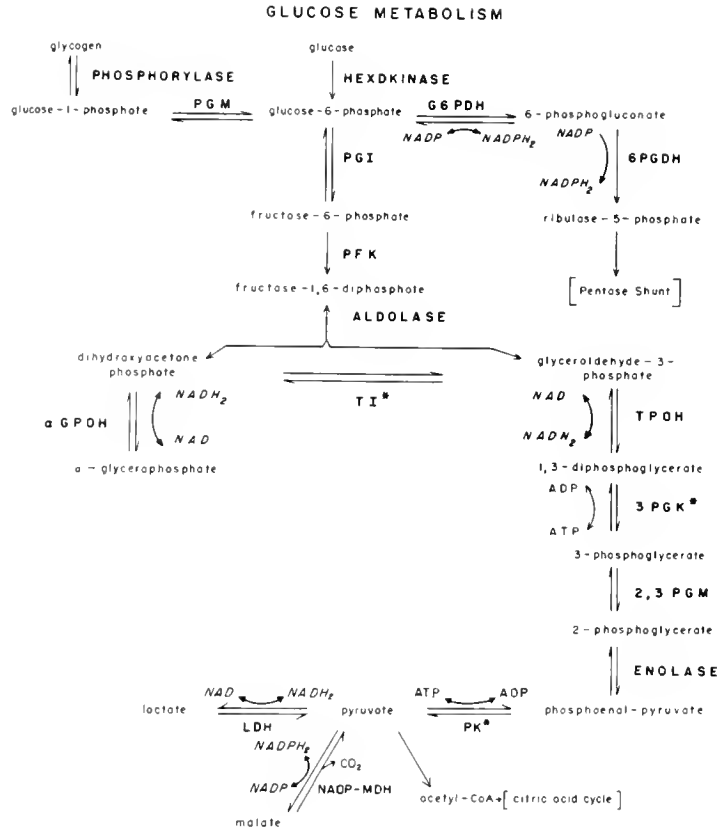

Fig. 1.

The glycolytic pathway. Under anaerobic conditions pyruvate is converted to lactate with the concomitant oxidation of $NADH_2$ to NAD. (From Markert, in *The Harvey Lectures*, Series 59, 187, 1965; reproduced with permission of Academic Press.)

isozymes. In 1959 a very sensitive and convenient method for analyzing LDH in tissue homogenates was developed by Markert and Møller (1). They coupled starch gel electrophoresis with a histochemical staining procedure for visualizing dehydrogenases. This facilitated the clear demonstration of tissue, ontogenetic, and species specificity of LDH isozyme patterns as well as the isozymes of a variety of other enzymes. With these data the biological significance of isozymes received general recognition As more data were accumulated it became apparent that most mammalian tissues contain five principle LDH isozymes and that the electrophoretic mobility of isozymes exhibits a high degree of specificity, as illustrated in Fig. 2.

Although there are readily discernible differences in electrophoretic mobility among the homologous LDH isozymes of widely different vertebrate species, it is observed that the overall isozyme patterns of homologous tissues are remarkably similar. This is clearly illustrated by comparing the LDH isozyme patterns of a given tissue, such as heart muscle, from several species as in Fig. 3. In general, vertebrate heart muscle is richest in the more rapidly migrating isozymes, LDH-1 and LDH-2, while vertebrate skeletal muscle is richest in LDH-5 and LDH-4. At the present time, the major apparent exceptions to this generalization are found among the fishes. For example, as shown in Fig. 4, the heart muscle of the whiting exhibits a remarkably bizarre pattern of LDH isozymes which is difficult to interpret. A few other exceptions should be noted. Skeletal muscle

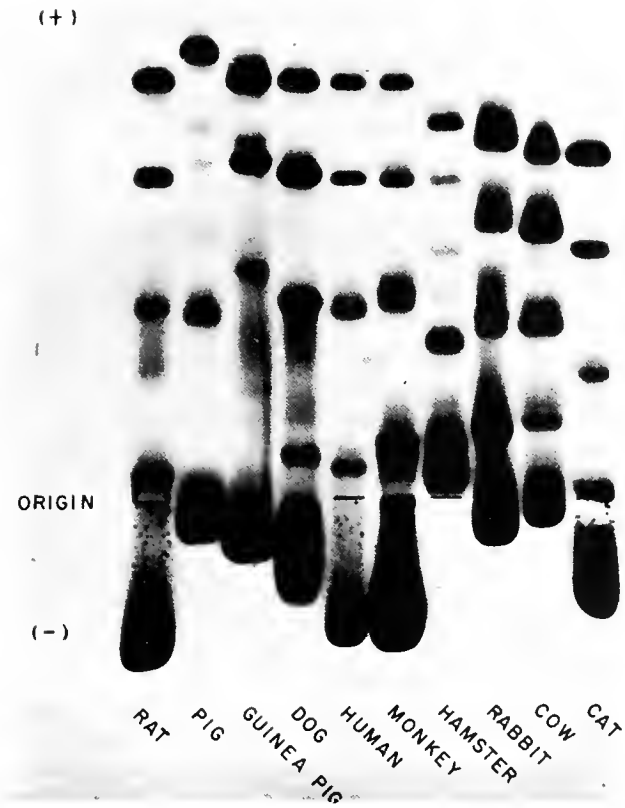

Fig. 2.

Zymogram of the isozyme patterns of mixed tissue homogenates from 10 mammalian species. Note the differences in electrophoretic mobility of homologous isozymes among the different species. LDH-1 is the fastest or most anodally migrating (most negatively charged) band. The slower moving bands, possessing a progressively decreasing negative charge, are designated LDH-2, LDH-3, LDH-4, LDH-5, respectively. In our electrophoretic system, LDH-5 is essentially neutral and its apparent cathodal movement is the resultant of electroendosmosis within the electrophoretic medium (starch gel). (From Markert, in *The Harvey Lectures*, Series 59, 187, 1965; reproduced with permission of Academic Press.)

which is capable of indefinitely sustained ("heart-like") activity, such as the breast muscles of certain birds (8), and, as we have recently observed, the flight muscles of bats (9), contains a predominance of rapidly migrating LDH isozymes. The similarities in LDH isozyme patterns among homologous tissues are emphasized by the differences in the isozyme patterns among heterologous tissues. This is

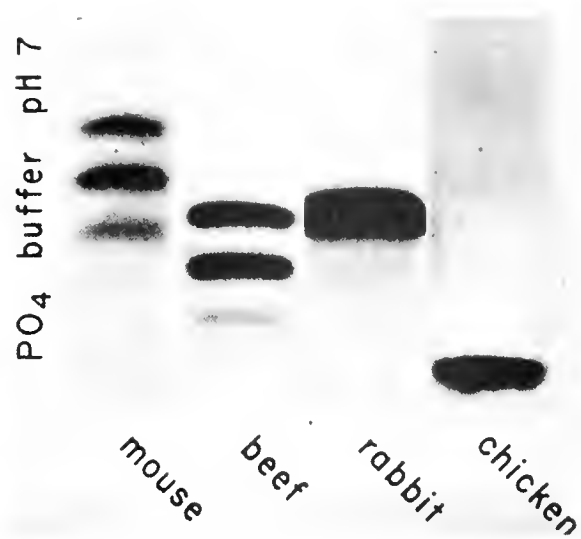

Fig. 3.

Zymogram demonstrating the similiarities in LDH isozyme pattern of heart tissues from 4 different species: the mouse, cow, rabbit and chicken. Heart muscle LDH from most vertebrate classes consists mainly of the more anodally migrating isozymes, LDH-1 and -2.

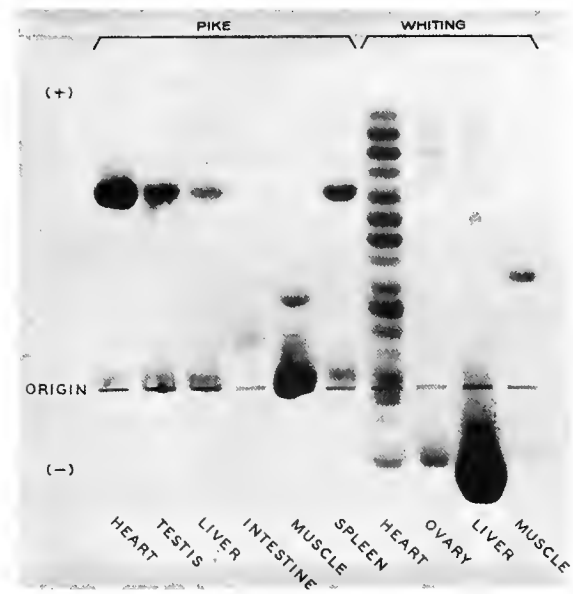

Fig. 4.

Zymogram of the LDH patterns of representative tissues of the pike (left) and the whiting (right). Both fish are members of the order Osteichthys. Note the complex pattern of isozymes found in heart tissues of the whiting.

illustrated by comparing Fig. 5, the isozyme patterns of various tissues of the Rhesus monkey, with Fig. 3, the isozyme patterns of the heart tissues of several other vertebrates.

The predominance of LDH-1 and LDH-2 in heart muscle and LDH-5 and LDH-4 in skeletal muscle suggests that the different isozymes have different physiological roles. In general, tissues possessing a highly aerobic metabolism (e.g. heart, brain, kidney cortex) contain mainly the most negatively charged isozymes, LDH-1 and LDH-2, while those tissues possessing a high anaerobic metabolism contain mainly LDH-5 and LDH-4. These observations seem to indicate that the net charge on an isozyme molecule may be important in determining its intracellular localization and may thus be a reflection of its metabolic role.

The stable isozyme pattern of adult tissues must have arisen at some time during development through a sequence of orderly changes. This has been clearly demonstrated by the direct analysis of tissues of the mouse at different stages of development (3) and is illustrated in Fig. 6 and 7. LDH-5 is the predominant isozyme in embryonic mouse tissues (Fig. 7). As development progresses the isozyme pattern migrates, in effect, toward the anodal end of the electrophoretic spectrum so that in most tissues an increasing proportion of enzyme activity becomes localized at the LDH-1 end of the spectrum. Only in those adult tissues, such as liver and skeletal muscle (Fig. 6), in which LDH-5 is the predominant isozyme, is the redistribution of enzyme activity during development relatively insignificant.

From these studies there was no indication that the isozyme patterns of the different tissues shift synchronously, and it is quite obvious that all do not shift to the same extent. However, the direction of the shift, when it occurs at all, is the same for all tissues. In some tissues, such as mouse heart muscle, the change in isozyme pattern was sufficiently rapid to exclude a corresponding change in cell population. Therefore it would seem that isozyme patterns must change within individual cells. It is interesting to note that LDH-1 is the predominant isozyme in embryonic birds and that during development, in contrast to the situation in mammals, the isozyme patterns shift toward the LDH-5 end of the spectrum so that in adults the LDH patterns in homologous tissues of birds and mammals are reasonably similar.

From the standpoint of the one gene-one enzyme hypothesis, the discovery of multiple molecular forms of enzymes presented perplexing problems concerning the genetic control of protein biosynthesis. To reconcile the phenomenon of isozymes with this hypothesis, it was proposed that a single gene controlled the synthesis of a single protein which could be folded into five alternative configurations each possessing a different net charge. In order to test this hypothesis an attempt was made in our laboratory to reversibly unfold and refold the LDH molecule. Beef heart LDH was treated with urea or guanidine-HCl to disrupt the hydrogen bonds maintaining the characteristic tertiary structure of the molecule. LDH is readily denatured and inactivated by these reagents but all attempts to reactivate the molecule by removal of the denaturing reagents were unsuccessful. A study of the nature of the products of denaturation was then undertaken. Denaturation of a preparation containing all five isozymes resulted in the appearance of only two protein bands following electrophoresis in acrylamide gel. During the denaturation procedure, three bands had disappeared. This unanticipated result combined with sedimentation data opened the door to our present understanding of the structure of LDH. From previous ultracentrifugal and other studies the molecular weight of native LDH had been calculated to be about 135,000 and the 5 major isozymes were all shown to possess identical molecular weights. However, when the guanidine denatured preparation was analyzed in the ultracentrifuge, the molecular weight was shown to be about 35,000 or approximately one-fourth that of the native protein. This data is summarized in Table I. The conclusion drawn from these results was that LDH exists in the native state as a tetramer composed of four equal sized (approx. M. W. = 35,000) subunits (10). As shown from acrylamide electrophoretic data these subunits exist as two electrophoretically distinct species designated *A* and *B* (11). It is obvious that random assortment of the two kinds of subunits into all possible combinations of four yields five isozymes of the composition shown in Fig. 8.

Several tests can be performed to verify the subunit hypothesis of LDH isozyme structure. This hypothesis assumes that LDH-1 consists entirely of *B* subunits, while LDH-5 consists of only *A* subunits. It follows that LDH-1 and LDH-5 must be distinct protein species. This could easily be verified by a complete amino acid analysis of each of these isozymes. Accordingly, both LDH-1 and LDH-5 were prepared in pure form by electrophoresis of crys-

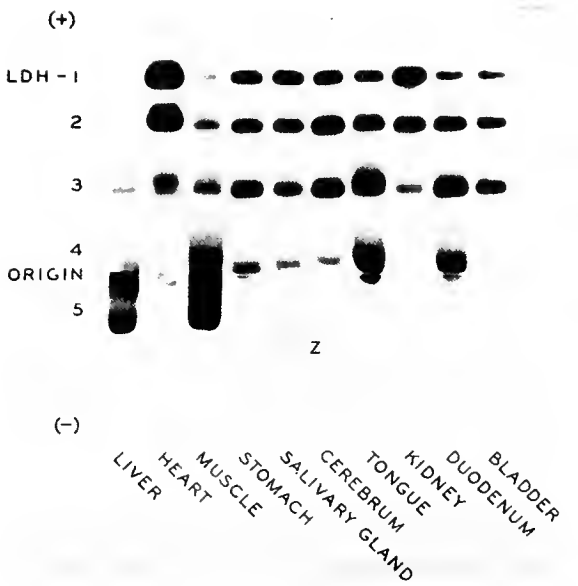

Fig. 5.

Zymogram of the LDH isozyme patterns of adult Rhesus monkey tissues. Each tissue is distinguishable by the relative proportions of the isozymes that it contains. Adult heart muscle is rich in LDH-1 and -2 while the proportion of these fast moving ioszymes is strikingly reduced or essentially absent in adult skeletal muscle in which LDH-3, -4 and -5 predominate. Note, however, that all five of the major isozymes are present in most tissues albeit in different proportions. (From Markert, in *The Harvey Lectures*, Series 59, 187, 1965; reproduced with permission of Academic Press.)

talline preparations which contained several of the isozymes. The pure isozymes were subsequently hydrolyzed and their constituent amino acids were analyzed by the method of Moore, Spackman, and Stein (12). The results of such analyses, shown in Table II, establish unequivocally that from the standpoint of amino acid composition the two kinds of subunits are different proteins. In addition, in full accord with the subunit hypothesis, LDH-3 consisting of 2 *A* and 2 *B* subunits, was shown to have an amino acid composition intermediate between that of LDH-1 and LDH-5 (13, 14). The amino acid analyses also revealed that beef heart LDH-1 contains 128 arginine and lysine residues calculated on the basis of a molecular weight of 135,000. Consequently, denaturation followed by trypsin digestion would be expected

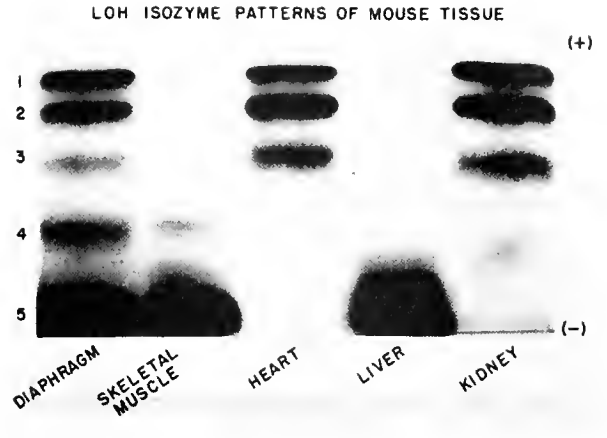

Fig. 6.

Zymogram of the LDH isozyme patterns of selected tissues of the adult mouse. Note that each of the tissues possesses a distinct proportion of the isozymes. Equal aliquots of total enzyme activity from each tissue were applied to the origin.

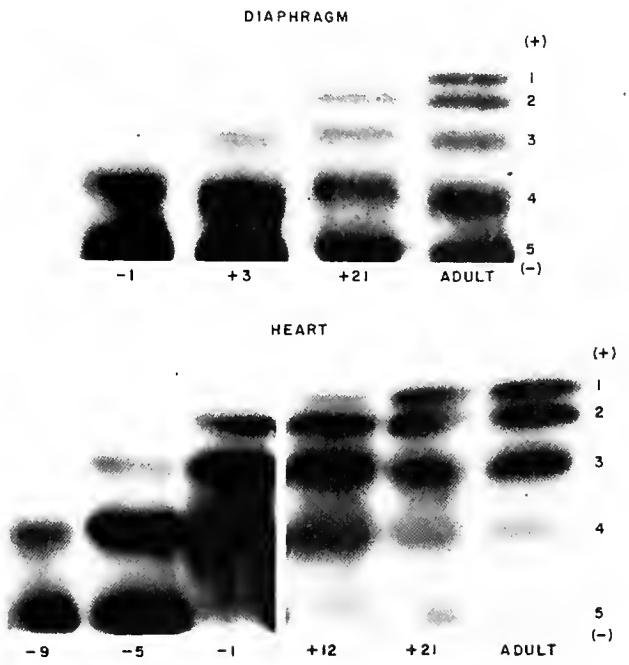

Fig. 7.

Zymogram demonstrating the shift in LDH isozyme patterns during development of representative tissues of the mouse. The negative numbers along the abscissa indicate days before birth and the positive numbers indicate days after birth. The numbers along the ordinate designate the isozymes. With time, there is an increase in LDH activity at the anodal end of the electrophoretic spectrum and a concomitant decrease at the cathodal end.

to produce about 128 peptides. The actual number of peptides found by this technique was about thirty or one-fourth the expected number. This result certainly reinforces the proposal that LDH-1 is a tetramer composed of four identical

TABLE I

Molecular Weights of Lactic Dehydrogenases Determined in the Multichannel Short Column Equilibrium Cell, Using Schlieren Optics

	Phosphate Buffer pH 7.2	Guanidine-HCl*
Beef Heart LDH-1	134,000	34,000
Beef Heart LDH-V	140,000	35,000
Pig Heart LDH-1	132,000	34,000

* a $\bar{v}$ of 0.740 has been assumed in all calculations.

TABLE II

Amino acid composition of LDH isozymes from beef muscle.

	Number of amino acid residues per molecule of enzyme	
Amino Acids	LDH-1	LDH-5
Lysine	94	95
Histidine	25	34
Arginine	34	52
Aspartic Acid	123	104
Threonine	56	62
Serine	92	61
Glutamic Acid	124	135
Proline	42	63
Glycine	91	100
Alanine	72	122
Valine	135	82
Methionine	32	20
Isoleucine	86	73
Leucine	130	118
Tyrosine	26	35
Phenylalanine	19	26

[1]Based upon a molecular weight of 135,000 (assuming 12 residues of cysteine and 30 residues of tryptophan in each isozyme).

LDH-1	LDH-2	LDH-3	LDH-4	LDH-5
↓	↓	↓	↓	↓
A^0B^4	A^1B^3	A^2B^2	A^3B^1	A^4B^0

Fig. 8.

Proposed subunit composition of the five major isozymes of LDH. LDH-1 consists entirely of *B* subunits and LDH-5 consists entirely of *A* subunits. The intervening isozymes, LDH-2, -3, and -4, are the various combinations of the *A* and *B* subunits.

subunits. Beef LDH-5 was subjected to the same type of analysis and also yielded about thirty peptides. A comparison of the peptide maps of LDH-1 and LDH-5 of beef revealed that some of the peptides were common to both of these ioszymic forms, but most were clearly different. It may be concluded from these observations that the *A* and *B* polypeptides are related, but long stretches of the primary structure must be quite different.

Perhaps the best test of a subunit hypothesis of isozyme structure is the dissociation of the active polymers into their constituent monomers and reassociation of the monomers into new active configurations. It was discovered in our laboratory that this can be readily achieved by freezing and thawing equal quantities of LDH-1 and -5 in neutral phosphate buffer which is one molar in NaCl (15). After thawing, an aliquot of the preparation is analyzed by electrophoresis in starch gel and subsequent staining of the gel slab for LDH activity. Preparations treated in this manner show all five isozymes in the proportions of 1:4:6:4:1, the expected binomial distribution of isozymes assuming the *A* and *B* subnits associated in a random manner.

In contrast to the irreversible denaturation obtained by treatment of LDH with urea or guanidine, the salt-freezing technique is quite mild. It seems possible that the subunits maintain their tertiary configurations essentially intact during this mild dissociative procedure. Although the salt-freezing technique is by far the most efficient method for recombining isozymes, saturated salt solutions in the absence of freezing as well as repeated freezing and thawing in buffer alone will gradually produce recombination. The recombination of LDH is not influenced by NAD and NADH, lactate, or pyruvate, and is independent, within wide limits, of the concentration of LDH. The optimum salt

concentration for attaining equilibrium recombination ranges from 0.1 to 4.0 *M*. Concentrations lower than 0.1 *M* are much less effective. It is of interest to note that only a few salts promote recombination and that both cations and anions play an important role in the process. Among the effective cations are sodium, potassium, lithium, magnesium, and zinc. Chloride, bromide, iodide, nitrate and phosphate are effective anions. Certain other ions, for example, borate, sulfate, and tris, inhibit recombination.

From elementary genetic considerations, since the *A* and *B* subunits of LDH are different proteins, they must be under the control of different genes. Recent genetic evidence bears this out. An LDH mutant has been discovered in the deer mouse *Peromyscus maniculatus* (16). In these animals, the mutation occurred at the *B* locus, and, as theory predicts, the heterozygote produced fifteen isozymes. During the screening of several diverse human populations mutants were found at either the *A* or *B* loci (17). To our knowledge, no double heterozygotes have yet been reported.

A third gene controlling the synthesis of a third type of LDH subunit, designated the *C* subunit, was discovered by Zinkham and coworkers (18). *C* polypeptides appear to be formed mainly (perhaps exclusively) in the sperm. Isozymes containing *C* polypeptides are responsible for the so-called "X-bands" of LDH activity found on zymograms of testis homogenates. In some mammals only one X-band is observed and it is assumed to be a tetramer of *C* subunits. Several X-bands have been detected in testis homogenates of other mammals. However, in these cases it has been shown that the additional bands are the result of the polymerization of *C* subunits with either *A* or *B* subunits (19). More recently, Zinkham and coworkers have shown that, in pigeons, the *C* gene exists in two widely distributed allelic forms designated *C* and *C'* (20). From testicular homogenates resolved by the technique of starch gel electrophoresis, they have been able to classify each pigeon into one of three phenotypic classes designated CC, CC', and C'C'.

Although it is theoretically possible to form fifteen isozymes from three different subunits no such number has been observed in sperm homogenates. The following interpretations of this may be brought forth. It is possible that the freedom of combination necessary for the formation of the fifteen isozymes does not exist or that the gene controlling the *C* polypeptide biosynthesis may be turned on only when the *A* and *B* genes are turned off. It is also possible that certain hybrid molecules cannot be formed for purely physical reasons or that certain hybrid combinations are inactive. However, a mixture of *A*, *B*, and *C* subunits will readily recombine *in vitro* to yield the expected fifteen different tetramers.

LDH zymograms of many different animals, especially the rabbit, show that several of the basic five isozymes exist as two or more distinct bands of enzyme activity (Fig. 9). An entirely satisfactory interpretation of the phenomenon of subbanding is not yet available although

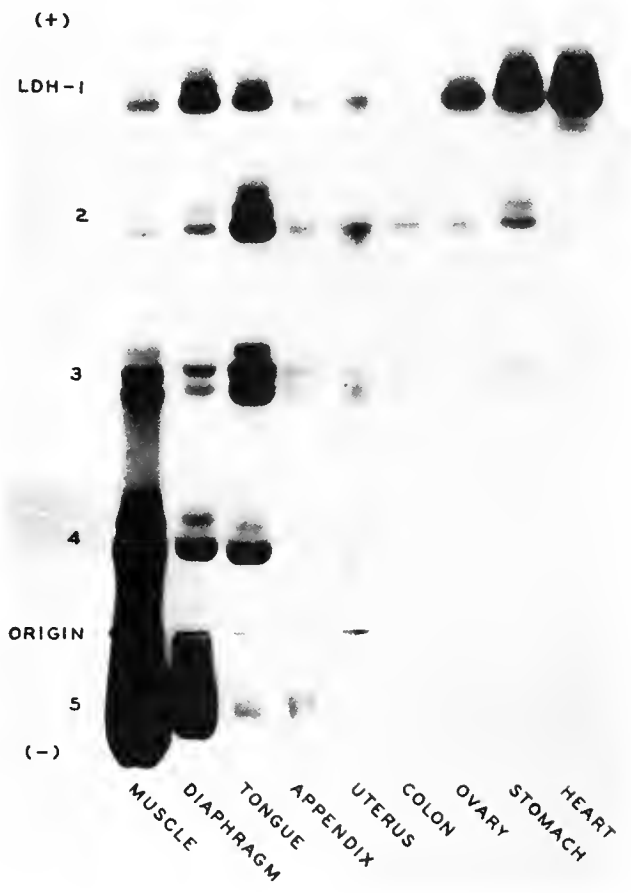

Fig. 9.

Zymogram of LDH patterns of various tissues of the rabbit. Note the multiple banding, termed subbanding, of most of the isozymes and the variation in subbanding which exists among homologous isozymes of different tissues. The subbanding is relatively constant for any particular species but varies considerably among different species. (From Markert, in *The Harvey Lectures*, Series 59, 187, 1965; reproduced with permission of Academic Press.)

several hypotheses have been considered. Among these is the proposal by Fritz and Jacobson that the subbanding in mouse tissues is the result of the differential binding of NAD by the subunits (21). The possibility that a small molecule such as NAD, by becoming attached to the subunits, can change the net charge, and hence the mobility of an isozyme, is certainly not unreasonable. However, this hypothesis was not supported in identical experiments with rabbit LDH. Another interpretation proposes that subbands represent permutations of the tetrameric combinations. This is supported by the observation that the mixing and recombination of rabbit LDH-1 and LDH-5, which in themselves show no subbanding, yields subbanding at the LDH-3 position. Kaplan and Costello have advanced the hypothesis that the subbanding in mouse LDH results from the existence of two different *A* subunits each of which is under the control of a separate gene (22). This interpretation is strongly supported by numerous observations of the existence of the subbanding in the pattern predicted for two different *A* subunits. The presence of such patterns in inbred strains of mice rules out heterozygosity as an alternative and suggests the existence of a fourth gene controlling the synthesis of LDH polypeptides. Clearly no single one of these interpretations fits all of the data. Indeed, there may be no all-encompassing explanation.

The existence of the X-bands and subbands tends to emphasize the fact that starch-gel electrophoresis resolves mammalian LDH into five major zones of activity. However, the isozyme pattern can differ considerably among mammals (Fig. 2) and among different vertebrate classes, as shown in Fig. 10. It is of interest to note that the net charge on the *B* subunit (the more negatively charged subunit) of mamalian LDH is apparently greater than that on the homologous subunit of most other vertebrate classes as reflected in the greater mobility of mammalian LDH-1. However, the *A* and *B* subunits of the vertebrate classes, excepting some fish, must be remarkably complementary in that most can be combined to form functional hybrid molecules (tetramers) of LDH by means of the salt-freezing technique as illustrated in Fig. 11.

PAPACONSTANTINOU: Aren't there more than five bands in the sixth column from the left? Yet you started out with pure LDH.

MASSARO: Yes, there are more than five bands. I started out with pure beef LDH-1 which was hybridized with rattlesnake muscle LDH and electrophoresed. Muscle of this species of rattlesnake contains several LDH isozymes, seven, in fact.

PAPACONSTANTINOU: Then are the complementary LDH-1's combining?

MASSARO: Yes, however when complementary LDH-1's are hybridized, if they have very close mobilities, the hybrid isozymes do not separate into distinct bands in our electrophoretic system.

Let us divert for a minute to a fish story. We have studied, to date, approximately thirty species of fish and have found that they can be placed conveniently into three categories according to the number of isozymes of LDH that they possess and the hybridization characteristics of these isozymes. Those fish possessing a single band of LDH activity, as revealed by starch gel electrophoresis, are placed in one category. This group consists of the fluke and related flatfish. In another category are place those fish possessing either two or three bands of LDH activity. There are some twenty-plus species in this group, evenly distributed between the two and three banded varieties. The third category consists of those fish possessing more than three bands of LDH activity. So far we have placed only three species in this group, the herring *(Alosa aestivalis)*, the shad *(Alosa sapidissma)*, and the whiting *(Merluccius bilinearis)*.

Under our conditions, any two of the LDH isozymes of the herring will readily hybridize with one another to form the expected autogenous hybrid molecules. This is also true for the isozymes of the whiting, and the shad (i.e., those fish possessing three or more bands of LDH activity). The fluke, having only one band of LDH activity, obviously does not show autogenous hydridization. All four of these species will also form hybrid molecules with one another and with mammalian LDH. Significantly, those species possessing two or three bands of LDH activity will not form autogenous hybrid molecules although they will hybridize with mammalian LDH and LDH from the two other groups of fish. The factors underlying the lack of autogenous hybridization within this group are under investigation in our laboratory.

Another interesting aspect of this study was the discovery of a very rapidly migrating band of LDH activity in the eye of many species of fish. This band has a mobility greater than that of mammalian LDH-1 and, like the *C* tetramers of sperm LDH, may represent another type of LDH isozyme.

Further evidence of the remarkable com-

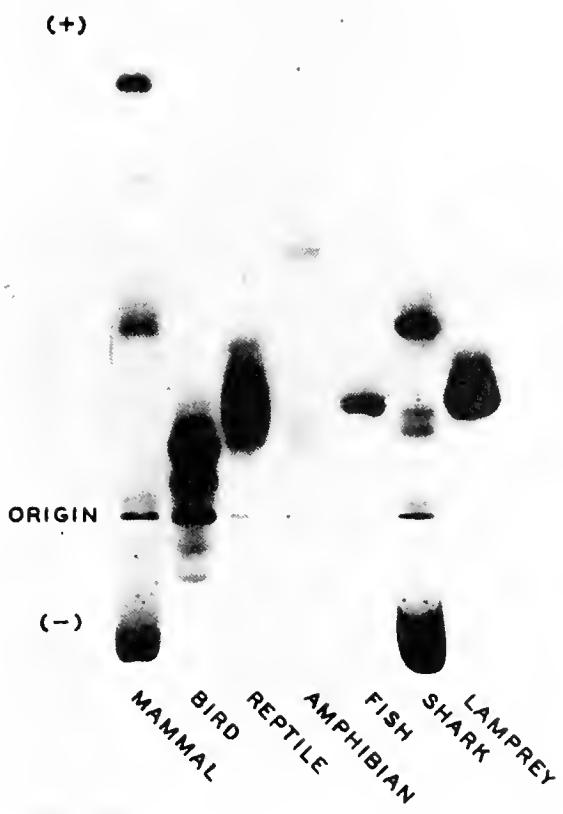

Fig. 10.

Zymogram of the LDH pattern of a representative of each of the classes of vertebrates. The representative species are human (mammal), Adelie penguin (bird), rattlesnake (reptile), Amphiuma (amphibian), fluke (bony fish), sand shark (cartilagenous fish), and the lamprey. Note the considerable variation in the number and mobility of LDH isozymes present in each of these organisms. (From Markert, in *The Harvey Lectures*, Series 59, 187, 1965; reproduced with permission of the Academic Press.)

plementarity of the subunits of vertebrate LDH is presented in Fig. 12, which shows the isozyme patterns obtained when horse LDH is hybridized with lamprey, fish, or salamander LDH. These patterns are relatively simple. More complex patterns are obtained in hybridizations involving other organisms. This is illustrated in Figs. 13 and 14 in which the results of the hybridization of chicken LDH with horse, snake, cow, or rabbit are shown.

TILL: Why are you doing all this?

MASSARO: For kicks! Seriously, one of our major interests is finding out how the LDH tetramer is put together. We feel that a study of various aspects of the phenomenon of interspecific hybridization is a valid approach to the problem.

McCARL: Do you always get the same patterns of hybridization?

MASSARO: Yes, they are very constant between any two given species.

It would be expected that the catalytic properties of the hybrid molecules differ from those of the parental types. This is analogous to the situation encountered with the heteropolymeric isozymes formed by recombination of LDH-1 and -5 from the same species. From our data, it appears that, in closely related animals,

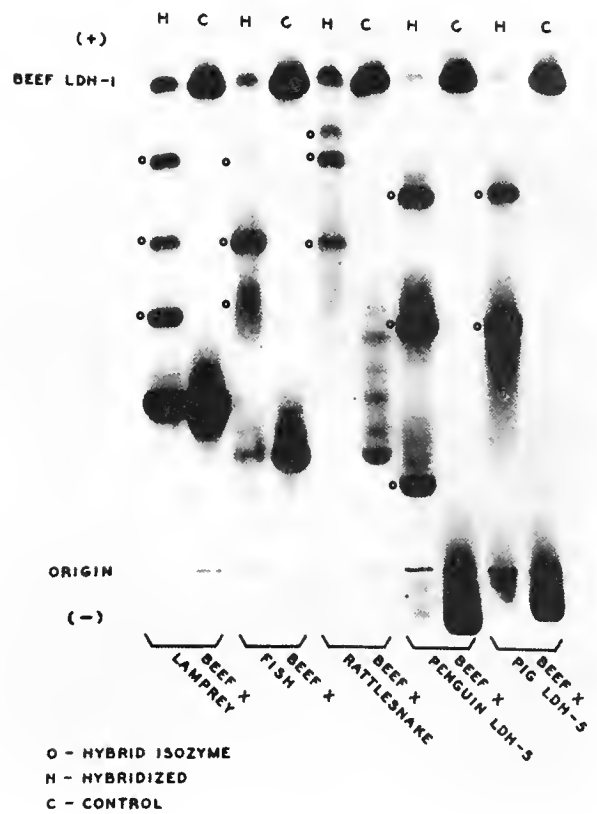

Fig. 11.

Zymogram illustrating the hybrid isozymes of LDH formed between beef LDH-1 and LDH from several classes of vertebrates (lamprey, fish, rattlesnake, penguin LDH-5, and pig LDH-5). The hybrids are indicated by small circles. (From Markert, in *Ideas in Modern Biology*, 1965; reproduced with permission of the National Academy of Sciences).

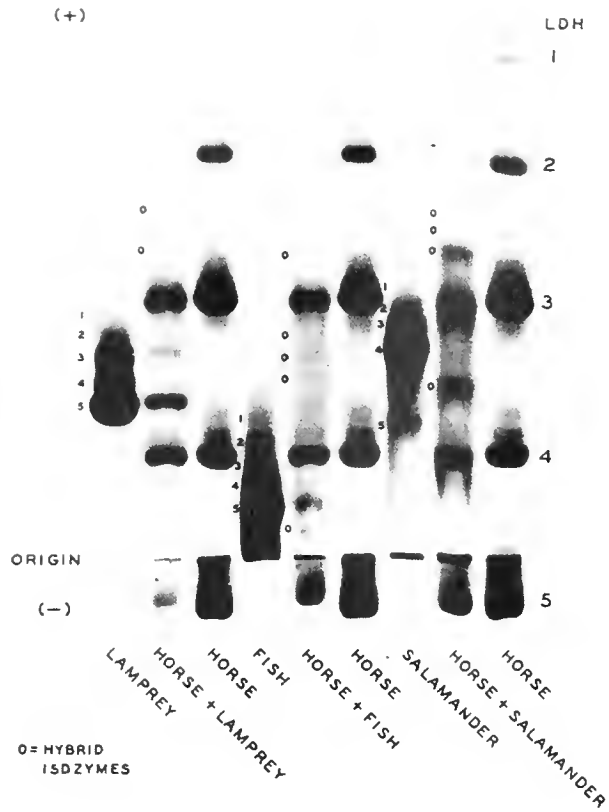

Fig. 12.

Interspecific hybridization of horse LDH. The isozymes of native LDH are designated by the numbers 1, 2, 3, 4, 5, while the hybrid isozymes are designated 0. Fish refers to the herring, *Alosa aestivalis*, and salamander to the newt, *Diemictylus viridescens*. (From Markert, in *The Harvey Lectures*, Series 59, 187, 1965; reproduced with permission of Academic Press.)

Fig. 13.

Interspecific hybridization of horse LDH. Chicken refers to White Leghorn chicken heart LDH and snake to the LDH of pooled tissues of the diamondback rattlesnake (*Crotalus adamanteus*). The numbers along the ordinate designate the isozymes of horse LDH.

interspecific hybrid molecules have catalytic properties analogous to those of the intraspecific hybrid molecules. However, as the evolutionary distance between species increases, the enzymatic activity of the hybridized preparation decreases. This loss in activity may be due to the formation of completely or partially inactive polymers.

Mammalian LDH-1 (the tetramer composed of *B* subunits) will combine with LDH-5 (the tetramer composed of *A* subunits) from any of the other six vertebrate classes to form at least three hybrid molecules. In an identical hybridization in which there are two kinds of *B* subunits with respect to charge, theoretically, fifteen isozymes can be formed. If, in addition, two differently charged *A* subunits are involved, then thirty-five different isozymes should be formed. However, the resolution of thirty-five isozymes may exceed the capabilities of the starch-gel electrophoretic system. In any event, as many as twenty-five distinct bands have been counted on the zymograms of certain hybridizations such as between chicken and horse.

The occurrence of isozymes of LDH in nearly all vertebrates which have been examined strongly suggests that, for certain enzymes, multiplicity of form is evolutionarily advantageous and does not represent simple heterogeneity of no biological value. The implication is that the individual isozymes subserve a specialized role in the economy of the organism. This is supported by the fact that, although all isozymes of LDH catalyze a characteristic

chemical reaction, they possess markedly different physical and chemical properties. In the light of this evidence, it seems reasonable to conclude that isozymes are groups of molecules of common origin that have become differentiated to meet highly specific requirements within the cell. The specialization of the individual isozymes indicates that they may be located in different places within the cell, or concentrated in different kinds of cells and tissues. Evidence in this regard has been brought forth by several investigators (23, 24, 25).

An insight into the physiological role of individual isozymes has been provided through determinations of their optimal substrate concentrations. The earliest investigation, by Plagemann *et al.*, revealed that the optimal pyruvate concentration for human LDH-1 is considerably lower than that for human LDH-5 (26). This has now also been established for LDH-1 and LDH-5 from other vertebrates (27). For example, in a series of experiments carried out in our laboratory (summarized in Fig. 15), it was observed that the optimal pyruvate concentration of horse LDH-1 is distinctly lower than that of horse LDH-5. In the case of the fluke whose tissues reveal only a single band of LDH activity as analyzed by starch gel electrophoresis, both heart and skeletal muscle LDH appear to have identical substrate optima. This and other data (28) indicate that fluke heart LDH is identical to fluke skeletal muscle LDH. Since the pyruvate optimum of fluke LDH is similar to that of horse LDH-5, it seems reasonable to assume that other properties of fluke LDH would be similar to vertebrate LDH-5 and that fluke LDH is, in effect, an LDH-5.

These observations are significant in that LDH-5 is found mainly in tissues, such as skeletal muscle, which are subject to periods of relative anaerobiosis and consequently are subjected to relatively high concentrations of pyruvate and lactate due to an increased functioning of the glycolytic pathway and decreased functioning of the tricarboxylic acid cycle. On the other hand, LDH-1 is found mainly in well oxygenated tissues with a high aerobic metabolism such as heart and brain in which high concentrations of pyruvate and lactate are not encountered.

An interpretation of this data involves the effect of high concentrations of lactate on muscle tissue. As is well known, during violent exercise, lactate can accumulate in skeletal muscle until the muscle is paralyzed. Obviously, this cannot be allowed to occur in heart muscle. The inhibition of heart muscle LDH at relatively low concentrations of pyruvate, then, acts as a check valve which functions to shunt pyruvate into the

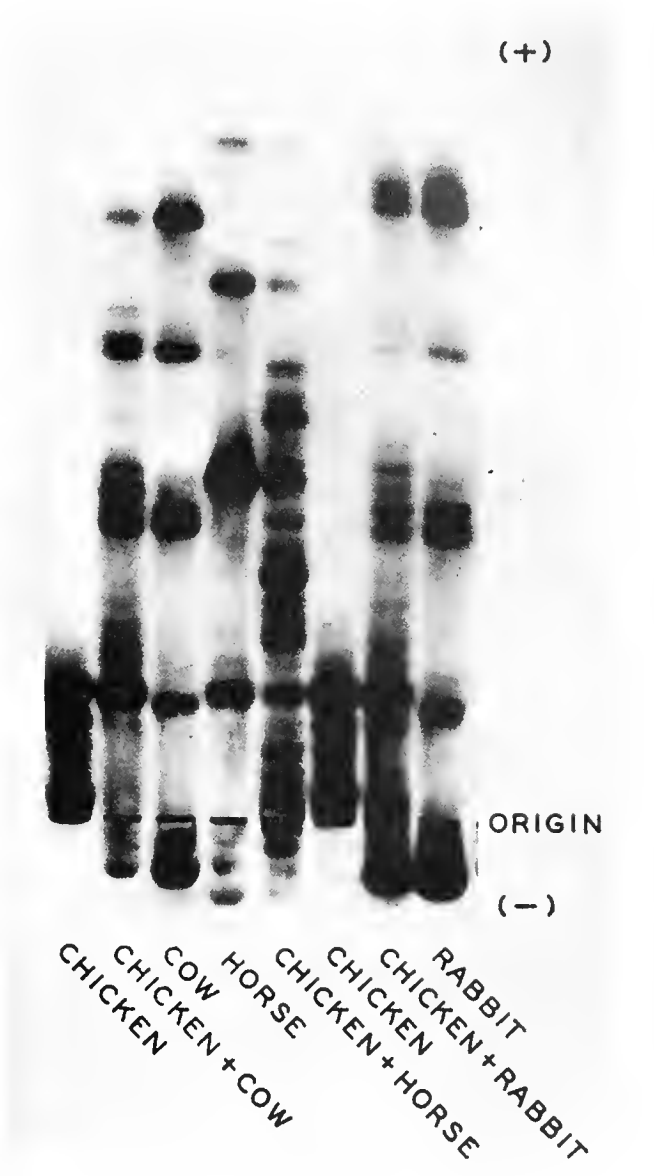

Fig. 14.

Interspecific hybridization of chicken LDH. The hybridizations were performed with LDH obtained from pooled tissues of each of the organisms. Note the complexity of these hybrid patterns as compared to those illustrated in Fig. 12. (From Markert, in *Ideas in Modern Biology*. 1965; reproduced with permission of the National Academy of Sciences.)

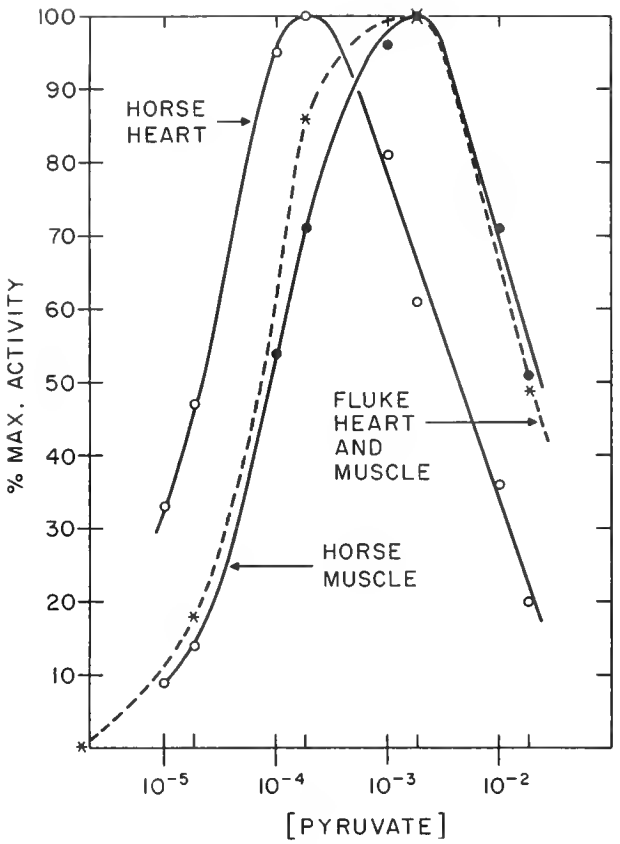

Fig. 15.

Pyruvate inhibition curve for horse LDH-1 and -5 and fluke LDH-1 and -5. Experiments were performed at 23° C in 0.1 *M* sodium phosphate buffer, pH (apparent) 7.0. The optimal pyruvate concentration for horse LDH-5 is higher than that for horse LDH-1. Fluke heart and skeletal muscle LDH, which are electrophoretically indistinguishable, have identical pyruvate optima. These optima are similar to that of horse LDH-5.

Krebs cycle so that lactate cannot accumulate in heart muscle.

As previously mentioned, this correlation extends to embryonic life. The tissues of mammalian embryos which have a relatively poor oxygen supply contain large amounts of LDH-5 whereas the well-oxygenated tissues of avian embryos contain mainly LDH-1.

A fundamental aspect of the interconversion of pyruvate and lactate as catalyzed by LDH is the oxidation-reduction of nicotinamide adenine dinucleotide. And this may be the most important function of LDH. The maintenance of the proper ratio of oxidized to reduced NAD is of considerable importance in that NAD is involved in numerous metabolic ractions.

In conclusion then, since the intracellular environment must surely vary from place to place from time to time within the cell, the existence of a spectrum of functionally distinct types of a particular enzyme would allow for a more efficient and precise control of a metabolic step. Since the discovery of the isozymes of LDH more than 100 other enzymes have been shown, at least tentatively, to exist in isozymic form.

DEERING: Do the isozymes of LDH always exist with four subcomponents?

MASSARO: Yes. So far as we know.

DEERING: Is this also true of some of the enzyme systems other than LDH? Are there always four or do you, perhaps, get combinations of three subunits there? Is there any reason to expect that they can't exist as dimers or trimers in some systems?

MASSARO: Isozyme systems other than the LDH system may be constructed on a dimer or trimer basis. The isozymes of MDH, malate dehydrogenase, for example, are dimers.

DEERING: You mentioned when you went through it the first time that the whiting pattern was very complex. Can you explain it in terms of *A*, *B*, and *C* subunits?

MASSARO: This is quite possible. However, we have not yet attempted the analysis. The banding pattern in this fish may be related to the subbanding in rabbit LDH. The multiple banding may have something to do with permutations of the tetramic structure of the individual isozymes. Such permutations could conceivably change the electrophoretic mobility of the ioszymes resulting in the very complex pattern that we find.

CANTINO: I have a question about the fish story in general. Do you work exclusively with frozen fish or freshly caught fish or mixtures of the two?

MASSARO: We use both fresh and frozen fish and never mix them unless we are certain that freezing has had no effect on the LDH isozyme patterns.

CANTINO: You stressed the importance of freezing and thawing upon recombination.

MASSARO: For the most part, in intact tissues, and let me stress intact tissues, not homogenates, LDH is quite stable. Intact tissues can usually be frozen and thawed without altering their LDH patterns. In a very few cases, however, we have seen entirely different patterns between frozen fish and fresh fish and, I am sure, this can also occur with tissues from other animal species. Some tissues we have studied could not possibly have been obtained fresh. For example, we have ob-

tained whale tissues from Alaska and penguin and seal tissues from Antarctica. From our experience, however, we feel confident that we are looking at essentially unaltered LDH patterns in frozen tissue.

CANTINO: Does it ever happen that you get a change in pattern?

MASSARO: If you mean, "can the pattern be changed experimentally?," there is some evidence in the affirmative. Kaplan's group appears to have done this in tissue culture by varying oxygen tensions.

PAPACONSTANTINOU: The point with oxygen tensions was that they didn't get any changes unless they used abnormally high oxygen concentration. You never would find that in living tissue. Isn't that about right?

MASSARO: That is true.

LOVETT: May I ask a rather naive question, perhaps? Has anyone looked carefully at some of these purified isozymes from the point of view of small molecules that might be functioning in a regulatory sense, at different stages differently? For example, in an embryo as compared with an adult?

MASSARO: Not that I know of.

LOVETT: There might be something like intermediates of other pathways, or some other coordinating system. I'm thinking about function. For example, how rapidly can it turn over?

MASSARO: To my knowledge, nothing has been done on this particular aspect of the problem.

GRUN: I had the impression that the idea was that there were two genes and that the tetramers you got were random combinations of polymers of these two genes. If *A* were active, you'd expect to find only, or mostly, LDH-5. If *B* were active, you'd expect the reverse of this. Some of the patterns that were on your figures made it look as though there wasn't a maximum at one end tapering off to the other end, as though there was something missing.

MASSARO: Well, you have to keep one thing in mind when you work with these zymograms. Each of these isozymes, LDH-1, LDH-5, and -2, -3, and -4, has different kinetic properties. When they are placed together into a reaction mixture which has a certain level of substrate and allowed to react, those having a higher turnover rate are going to show up as bigger, heavier blobs than those which have a very slow turnover rate.

PAPACONSTANTINOU: How much variation in turnover rate is there?

MASSARO: There is about a two-fold difference between beef LDH-1 and -5.

GRUN: Are -2, -3 and -4 intermediate?

MASSARO: Present evidence suggests that the properties of these heteropolymers reflect the proportions of the parental monomers which they contain.

DEERING: Do you always find that you get the proper ratios of isozymes if you know the relative amounts of *A* and *B*? Take a situation in which the amounts of *A* and *B* are not equal; you wouldn't expect a 1:4:6:4:1 ratio in that case. If you get the actual amounts of *A* and *B* you can always predict the amounts of 1, 2, 3, 4, and 5, or is there the possibility of some active mechanism which skews this in one direction or another?

MASSARO: Theoretically you can predict the distribution of isozymes, all things being equal.

TS'O: At least, Kaplan thinks so.

DEERING: It's merely a function of the concentration of the two?

MASSARO: It looks that way. However, *in vitro* the distribution can be skewed by unknown factors.

PAPACONSTANTINOU: Under all situations the recombination seems to follow the binomial theorem. The reason you get the different combinations may be because one is turning over a thousand times more rapidly than the other. At least, my impression from Markert was that they never had any conditions under which they didn't follow recombination explainable by the binominal theorem.

MASSARO: I don't really think that this is worth pursuing to any great length.

GROSS: You look at a zymogram and you see a gene product; your conclusions about the amounts of these gene products depend on the rate of the reaction in the gel. Has anyone ever measured how much LDH-1, LDH-2, etc., are present in a homogenate? The question that's implied by this is, does the difference that you see in an isozymic pattern really reflect the difference in quantity?

MASSARO: If we know the turnover rates of the isozymes under our conditions, it does. This is the big problem. Of course, one way to find out is to resolve the isozyme mixture by electrophoresis, cut out the individual isozymes and measure the quantities and turnover number of each. If you get good recoveries for each isozyme you have the answer.

Now, we have done this. Unfortunately, this kind of analysis is usually unsatisfactory if

starch gel is employed because recoveries from starch gel are ridiculously low. Recently we have been working with an acrylamide gel system which is quite satisfactory. From the limited data which we have obtained I would say that there seems to be a reasonably good relationship between what you see and the quantities present in the original mixture.

GROSS: Are the genes contiguous?

MASSARO: We don't know.

EPEL: Are the shad, herring, etc., a group of fishes that are in the same evolutionary family?

MASSARO: Yes.

J. WRIGHT: In terms of the evolutionary scale, I think there's no pattern. My student, Novak, did a survey of LDH in various tissues in various species and among those, gar and bowfin are supposedly the most primitive. We get 5 bands for the gar and only two bands for the bowfin in almost all tissues looked at. In contrast, the perch and bass would be further up on the scale, and these have low numbers of bands and it varies considerably.

GROSS: Are these stages samples of these species?

J. WRIGHT: Yes, and there are individual differences within some of these species.

ZIMMERMAN: I just wonder how you can explain the two bands in some species. Is this explained in terms of an *A* and a *B*? Don't you need a minimum of 5 bands?

MASSARO: The structure of the isozymes of those species possessing two or three bands of LDH activity has not been worked out. It is conceivable, but improbable, that the LDH molecule of these species is a dimer; if so, one would not expect to find 5 bands of activity. Also, it does not necessarily follow that tetrameric molecules will produce five bands of activity since certain combinations of monomers may not be allowed.

TS'O: Did you study the mammalian case? Do you know whether these subunits have to function co-operatively or can each individual subunit function separately?

MASSARO: We don't know, but we are in the process of attacking this problem.

EPEL: Relating to what forms exist *in vivo*, perhaps, in breaking up the cells you're selectively causing some compartmental exchange?

MASSARO: That is possible.

EPEL: If you take tissue which specifically has LDH-5 and one which has LDH-1, mix the two homogenates together and then do a zymogram, do you just get 1 and 5 or do you get intermediates? Do you have to salt-freeze to get hybridization?

MASSARO: In our experience you have to either salt them heavily and freeze them or salt them tremendously with a very high concentration of salt and let them sit around for a long time before you'll get any hybridization.

J. WRIGHT: What is the relationship of these movements on starch and acrylamide?

MASSARO: At comparable pH's and gel densities the movements are reasonably similar with the exception of LDH-5 which runs toward the cathode in starch gel under our conditions and toward the anode in acrylamide.

J. WRIGHT: How about the cathode area of insertion, now? Do you get LDH-5 moving backward in the area of insertion?

MASSARO: In starch, yes. Although, under our conditions, LDH-5 is negatively charged, a strong electroendosmotic effect propels it cathodally. In acrylamide you do not have an electroendosmotic effect so it moves toward the positive pole.

FERGUS: In regard to hybridizing, have any attempts been made to use some non-LDH protein?

MASSARO: Yes, we tried it with MDH and IDH, but got no results.

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ANTIGEN SYNTHESIS DURING REORGANIZATION IN THE CELLULAR SLIME MOLDS

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Perhaps most of you are familiar with the details of the development of the slime molds. However, I'd like to emphasize certain steps in their development before continuing with the remainder of the talk. Figure 1 is a diagram of the development of two species of slime mold, *Dictyostelium mucoroides* and *Dictyostelium discoideum*. Aggregation of a homogeneous group of *D. discoideum* vegetative amoebae occurs, which, through morphogenetic movements, forms itself into a migrating pseudoplasmodium or slug. Further morphogenetic movement results in the formation of a mature sorocarp consisting of a small mass of cells supported by a slender stalk. Development is similar in *D. mucoroides* with the exception that *D. mucoroides* forms a stalk as it migrates. Eventually, a fruiting body is formed, again consisting of a mass of cells supported by a slender stalk.

If we examine a fruiting body of *D. discoideum* closely, we find that it has developed proportionally; that is, regardless of the size of the cell mass, about 70% of the cells differentiate into spores and the remaining 30% differentiate into stalk cells. The basis for this proportionality arises by the time of the migration stage. At this time two types of cells have differentiated: the so-called prespores and prestalks. Now, in *D. mucoroides* as stalk formation occurs continually during migration new prestalk cells are formed from the prespore mass. Thus, at any point during migration there is a constant proportionality between the prespore cells and the prestalk cells, which results in the formation of a proportional sorocarp.

The question arises, what is the mechanism involved in establishing this proportionality? Obviously it's a problem with the differentiation of two types of cells initially. More specifically, it's a problem in which two types of cells must

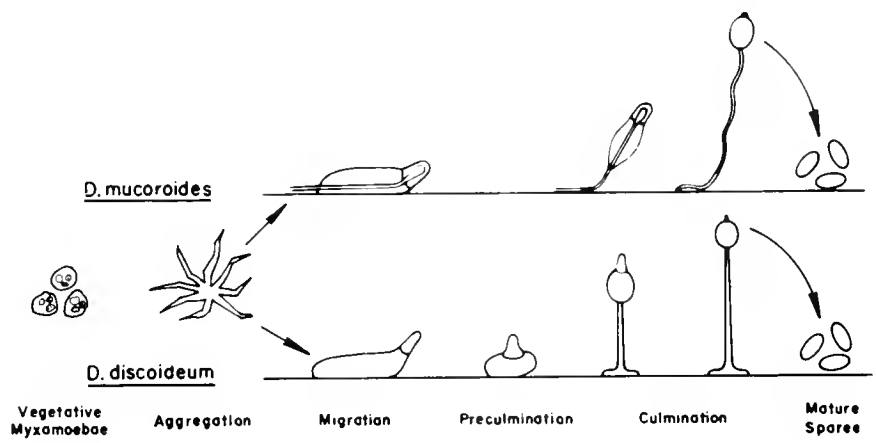


Fig. 1.

The developmental stages of *D. discoideum* and *D. mucoroides*.

differentiate in particular numbers.

One of the ways in which cell differentiation may be studied in these two slime molds is by immunological methods; and in this seminar today I want to talk about the use of fluorescent antibody in studying differentiation. This particular method was first employed by Takeuchi (1) in studies on *Dictyostelium*. This seminar is based upon a study which has recently been accepted for publication (2).

The first step in doing an immunological study involves the production of antisera (Table I). Antibody was produced to three species or strains of slime molds: *D. discoideum*, *D. mucoroides* (strain TYP) and a mutant of *D. mucoroides* isolated and reported by Filosa (3). These antisera were made to vegetative amoebae, migrating pseudoplasmodia and mature sorocarps, in each instance; that is, all three stages were used in producing the antisera of any one species. Now, the antiserum was conjugated with fluorescein iso-thiocyanate by more or less conventional means, the salient points of which involved the precipitation of gamma globulin by cold methanol, weighing of a small sample of the globulin solution with a microbalance in order to determine the total amount of globulins in the sample, and mixing the globulins with 0.0188 mg of fluorescein per mg of globulin (an amount we found to be optimum). Following conjugation at 5°C for 15 to 18 hours, the samples were centrifuged and then run through a Sephadex column to remove nonincorporated fluorescein from the labeled globulin. Such serum was used in staining various stages of the slime molds. Unless otherwise indicated homologous antiserum was used in the staining procedure.

Figure 2 shows *D. mucoroides* amoebae removed from an aggregating stream. We find that such cells, or such groups of cells, removed from the stream will stain with various intensities. Note the two extremes here: very dark cells which stained with little intensity and other cells which stained with a considerable intensity. I believe these correspond to the so-called "bright" and "dark" cells which Takeuchi (1) reported. I'll discuss the possible significance of these cells later on.

The early aggregates were sectioned at about 5 microns. Although bright and dark cells appear in the aggregating stream, once the cells aggregate to form a cell mass in the early aggregate the stain is more or less homogeneous

TABLE I

Preparation of Conjugated Antisera[1]

1. Gamma globulins precipitated from 1.0 volume serum by cold methanol. Reagents and fractionation procedure described by Dubert et al. (8).

2. Globulins redissolved in 0.85 volumes of 1.0% NaCl.

3. 100 µl aliquots of globulin solution dried and weighed, on Cahn ultramicro balance. Correction calculated for weight of NaCl in aliquot.

4. Globulin solution diluted with 0.15 volumes of 1.0 M carbonate-bicarbonate buffer at pH 9.0.

5. Globulin solution placed in 250 ml Erlenmeyer flask. Ice crystals produced in globulin solution by immersing flask in dry ice-methyl cellosolve bath (9).

6. In presence of ice crystals 0.0188 mg fluorescein iso-thiocyanate added per mg globulin and mixed with magnetic stirrer at 5°C for 15-18 hours (10).

7. Centrifuged 20 minutes at 3000 X G in refrigerated centrifuge to remove particulate matter resulting from conjugation.

8. Purification of fluorescein-conjugated globulins utilizing a G-25 fine Sephadex column (Pharmacia Fine Chemicals, Inc.) (11).

[1] From Gregg, 1965 (2), reproduced with permission of *Developmental Biology*, published by Academic Press.

(Fig. 3). You cannot detect that prestalk cells have differentiated at this stage. In the late aggregate prestalk cells begin to differentiate (Fig. 4). These prestalk cells are characterized by the fact that they tend to lose their cytoplasmic antigens. Consequently, they do not stain with high intensity. At the same time you see spots of intense staining in the prespore cells which mark the synthesis of prespore antigen. Consequently, all the cells in this area form prespore cells and the anterior cells which stain the least become prestalk cells.

Figure 5 shows a migrating pseudoplasmodium of *D. mucoroides*. This has been stained, however, with the normal conjugated antiserum. Little or no staining was found with normal conjugated serum. The preparation itself tends to transmit light in such a way that it appears to be bright, but the fluorescent staining is relatively low.

LOVETT: Is that region in the center the stalk?

GREGG: Yes. Figure 6 is another *D. mucoroides* slug stained with the antiserum. You can see that stalk formation is occurring; the stalk runs down through the center of the slime mold. The prestalk cells in the anterior area are fully differentiated now, resulting in the formation of a proportional slug. The prespore antigen increases in the prespore cells throughout the entire area. This results in a sharp delineation between the prespore cells and the prestalk cells. Thus, by this time these two types of cells have developed with the prestalk cells always in the anterior or leading end of the slug. The question arises, how does this polarity develop? Takeuchi has suggested that the bright-staining and dark-staining cells that he found - and that I have seen - in the aggregating streams sort out during aggregation. Simultaneously, the dark-staining cells lose even more of their staining and eventually end up in the anterior tip, thus composing the prestalk area. Consequently, the brightly-staining cells form the prespore area.

MASSARO: What is the magnification here?

GREGG: That's about 120X.

DEERING: What's that film along the edge of the slug in some figures? Is it something that peeled off?

GREGG: It's a slime track or slime sheath that's produced along the edges of the slime mold.

Now, it's possible that bright-staining and dark-staining cells sort out to form these two areas. However, the slug has developed proportionally, and in order to account for this we would have to assume that the prestalk and the prespore cells differentiated during the aggregation stage and aggregated in numbers suitable to form this proportionality in a cell mass of a certain size. It's a little difficult to conceive of this occurring. It would seem more obvious that proportionality results after the cells come together. However, since these cells can reversibly differentiate, it's possible that they differentiate in either direction, depending upon the necessity, in order for the proportionality to be established. Before continuing, however, in this discussion let's look at the situation in another system, *D. discoideum*.

TS'O: Excuse me for asking a question on the biology of this organism. Can you take a single cell and generate a mass like this or do you have to always start with lots of cells?

GREGG: Yes, it is possible. Either a single mature spore cell or a single amoeba will produce innumerable colonies.

In *D. discoideum* we have also been bright- and dark-staining cells in the aggregating stream. However, in the early aggregate we again see no evidence that the prestalk cells have differentiated (Fig. 7). This upper margin is what we call an edge effect, which you get in certain fresh preparations. This artifact does not represent the differentiation of prestalk cells.

Figure 8 shows a late aggregate of *D. discoideum*. This is the orientation of a late aggregate on an agar plate. They stand up just prior to flopping over and migrating about on the agar. Even at this relatively late stage one usually cannot see a differentiation of prestalk cells. On occasion there is a small tip end of prestalk cells which have differentiated, but otherwise the cell mass appears to be uniformly stained. It's obvious that the form and the polarity of the cell mass is independent of the differentiation of the prestalk cells. Thus, prestalk cells need not differentiate in order to produce this particular shape. Consequently, this suggests that subtle differences exist in the cell mass, prior to prestalk and prespore cell differentiation. Now, I suggest that one of these subtle differences is that of acrasin production which is at its greatest intensity in the anterior tip. Bonner (4) has shown this in *D. discoideum*. Perhaps such differences as this result in the differentiation of the cell according to the point at which it happens to be located.

Immediately after the late aggregate it is obvious that prestalk differentiation has oc-

curred (Fig. 9). Exactly what the mechanism is that caused the differentiation, of course, is a problem.

CHALKLEY: Is this a sharp or slow transition?

GREGG: If you look at enough of these, you can see small areas in the late aggregate that have begun to differentiate. Presumably, between the time they are standing up like this and the time they flop over they differentiate most of their prestalk cells. Now, it's impossible to say in this preparation how long this particular slug has been migrating.

KAHN: It might be worth pointing out that this process of tipping over takes no more than a few minutes.

GREGG: Yes. So differentiation may begin just prior to flopping and is completed in a relatively short time.

B. WRIGHT: Do you think this difference in staining intensity could be a difference in permeability to the stain?

GREGG: I don't think so because these are histological sections, of course, and I wouldn't think that cell permeability is involved here.

B. WRIGHT: Perhaps the spore cells have a more resistant coating.

GREGG: Since these are no longer whole cells, having been sectioned, I don't think a permeability factor could be involved.

LOVETT: You showed the two kinds of amoebae in the aggregating stream of *D. mucoroides*. Are they the same in this respect?

GREGG: Yes, they're present both in *D. discoideum* and *D. mucoroides*.

LOVETT: Can't you see them at all when it's still erect?

GREGG: You cannot distinguish two types of cells once the aggregate has formed. You can detect them only when you look at the separate amoebae taken from a late interphase or an aggregate.

LOVETT: If they're just lost in the mass, I wonder if they could creep up.

GREGG: Yes, and that is just the reason you cannot exclude sorting out. However, it's just amazing that by the late aggregate in *D. discoideum* you see very little evidence of prestalk cell differentiation. I would think that if sorting out was going to occur, it would occur as part of this process in elongating the cell mass. It's surprising, if it is one of the processes of slug formation, that you do not see more prestalk cell differentiation at this time.

KAHN: I want to ask another question about the staining. Are the classes of light and dark

Plate I. Figures 2 through 13

Fig. 2. *D. mucoroides* aggregating myxamoebae exhibiting different degrees of staining with homologous fluorescent antiserum (HFAS).

Fig. 3. *D. mucoroides* early aggregate exhibiting uniform staining throughout the cell mass (HFAS).

Fig. 4. *D. mucoroides* late aggregate exhibiting the initial differentiation of the anterior prestalk cells as indicated by the decreased cytoplasmic staining. Prespore antigen synthesis has started in certain cells of the posterior prespore area. Staining observed on all cell surfaces (HFAS).

Fig. 5. *D. mucoroides* slug exposed to homologous fluorescent normal serum. Staining is completely negative.

Fig. 6. *D. mucoroides* slug exhibiting intense staining with homologous fluorescent antiserum (HFAS) in the prespore area but lacking cytoplasmic staining in the prestalk and stalk cells. All surfaces show staining.

Fig. 7. *D. discoideum* early aggregate exhibiting uniform staining with homologous fluorescent antiserum (HFAS).

Fig. 8. *D. discoideum* late aggregate exhibiting uniform staining throughout the cell mass (HFAS).

Fig. 9. *D. discoideum* slug exhibiting intense staining in the prespore area but lacking cytoplasmic staining in the prestalk area. All cell surfaces show staining (HFAS).

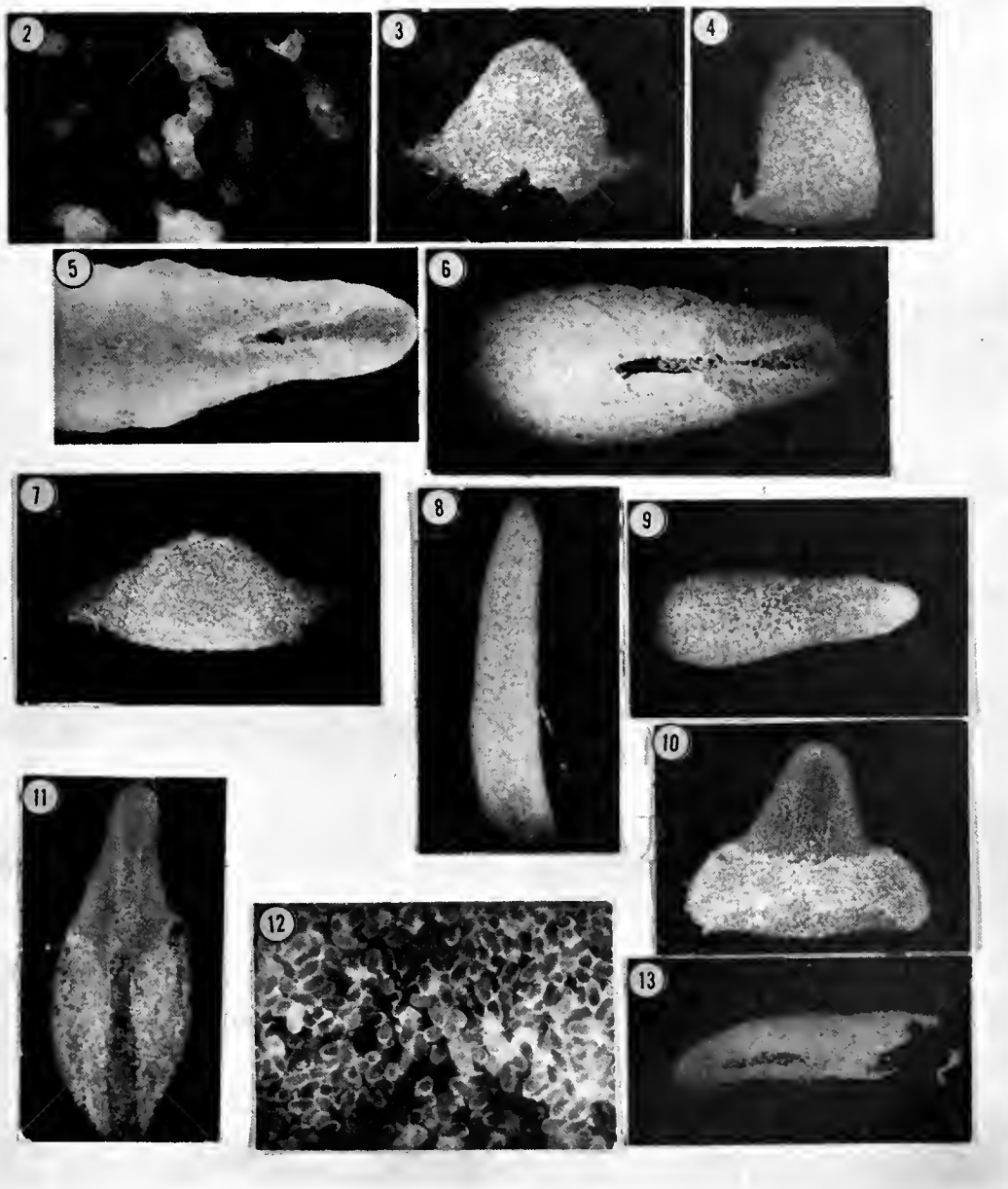
Fig. 10. *D. discoideum* preculm exhibiting an intense staining in the prespore area but negligible cytoplasmic staining in the prestalk, stalk and basal disc cells. All cell surfaces are stained (HFAS).

Fig. 11. *D. discoideum* culminating exhibiting a decreased intensity of cytoplasmic staining in the prespore area as compared to the preculm. Prestalk and stalk cells stained on cell surfaces (HFAS).

Fig. 12. Group of *D. discoideum* spores exhibiting a decreased degree of cytoplasmic staining as compared to preculm. Cell surfaces retain their staining capacity (HFAS).

Fig. 13. MV slug exhibiting the lack of well defined prestalk and prespore regions as noted in *D. mucoroides* slugs (*D. mucoroides* FAS).

(Figs. 2, 3, 7-12 from Gregg, *Devel. Biol. 12*, 377, 1965; reproduced with permission of Academic Press.)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cells absolute or do you have a graded series?

GREGG: In my opinion it is a graded series. The ones I pointed out were the extremes. Although photographs may be misleading I noticed in Takeuchi's black and white photos that there appear to be more darker-staining cells than bright cells. This is strange if these cells are going to sort out to form a slug with certain proportions.

KAHN: If you were to establish criteria for classifying light and dark cells and were to score the cells found in the aggregate do you think you would find the 30 (prestalk):70 (prespore) ratio?

GREGG: No, I don't think so. As a matter of fact, you might find various transitions and not necessarily this final proportion of very bright and very dark cells. You may find all intermediates in about equal proportions. Takeuchi has also referred to the various grades of staining caused by the granules in the cells.

KAHN: One final question. What was your antigen?

GREGG: All three stages injected into a rabbit. In theory we had antibodies to vegetative amoebae, slugs and mature stalks and spores. They were homogenized before being injected.

B. WRIGHT: How do you prepare these sections initially?

GREGG: They are fixed in Carnoy's and run through an alcohol series.

B. WRIGHT: Yes, but how do you kill them? What's the initial step? Do you freeze them?

GREGG: No, the fixation kills them. Carnoy's is essentially acetic acid alcohol and chloroform. These are paraffin sections.

B. WRIGHT: I see. Could this treatment itself differentially leach the two cell types?

GREGG: That's a possibility. However, Takeuchi used methanol and we have obtained identical results with these two methods.

GROSS: You're presumably looking at proteins with the fluorescence. Carnoy's fixer is 3:1 acetic acid and chloroform. It's a very effective protein fixer. It's unlikely that it would wash out antigens.

GREGG: This is more or less a conventional histological technique when using fluorescent antisera.

PAPACONSTANTINOU: Well, there's one thing that bothers me. Although it may be a little trivial here, I'd like to find it out. Dr. Deering asked you what was the staining material along the outside and you said it was slime. Is there any protein in that?

GREGG: The slime sheath could be polysaccharide.

PAPACONSTANTINOU: Well, would that stain?

GREGG: Perhaps the polysaccharides are antigenic.

PAPACONSTANTINOU: Oh, I see; you've got polysaccharide as well as protein antigens.

GREGG: Oh yes, that's very likely.

PAPACONSTANTINOU: You don't know whether that difference in staining is due to polysaccharide or to something else?

GREGG: No. That cannot be determined yet. I'm not sure exactly what the composition of the slime sheath is, but I would say it's probably polysaccharide.

ZIMMERMAN: Could you tell us once more how long it takes this thing to flop over?

GREGG: It's a matter of a few minutes. I'm quoting Dr. Kahn on this. At any rate, by the time the migrating pseudoplasmodium has formed, prestalk cells have developed such that this will result in the development of proportional fruiting bodies. Again you can see that one of the characteristics of prestalk cells, in both *D. discoideum* and *D. mucoroides*, is that they tend to lose their cytoplasmic stain. Consequently, the cytoplasmic antigen must be lost at the anterior end. Obviously development of the slime mold depends upon differentiation of these two types of cells, prespore and prestalk cells. How do we account for the loss of cytoplasmic antigens in these prestalk cells?

DEERING: I have one more point I'd like clarified. Is this a mixture of the antibodies to all three stages injected at the same time?

GREGG: Yes.

Figure 10 shows a preculmination stage. There is no drastic change in prespore staining in the preculmination stage. The stalk has begun to develop; the lack of cytoplasmic antigens is seen to continue in the prestalk cells.

DEERING: Is that the disc at the bottom?

GREGG: Yes, the basal disc has begun to form here, and it also loses its cytoplasmic antigens, and consequently loses its staining capacity.

KOHNE: Are the cells rapidly dividing as it's falling over?

GREGG: No, there is very little, if any, cell division once the aggregate is formed.

For those of you who are not familiar with the way the slime mold develops, the prestalk cells in this area move up and flow down into a funnel-shaped area formed by the stalk. The cells pile up on top of one another in the same process by which a chimney is formed, and this results in the raising of the spore mass.

EPEL: One point I don't quite understand is, if these are antibodies against all three stages, what is the mechanism of differential staining?

GREGG: You mean why is there no stain in the prestalk or stalk cells? Apparently the antigens are lost and when you build up antibodies, there are apparently no antigens present in here which are specific to build antibodies which would in turn stain these cells.

EPEL: In other words, these antibodies are differential against the various proteins.

GREGG: Yes. If you build up antibodies to all three stages, and for some reason these cells lose their antigens, or lose the antigens that they had at an earlier stage, the antibodies would not stain these cells because the antigens are gone.

KAHN: If they are antibodies against all cell types, why shouldn't the prestalk cells show the same staining response?

GREGG: They stain on the cell surfaces but not in the cytoplasm. If there are no antigens inside a cell, there is no reason to believe they would be antigenic.

KAHN: What do you see at a higher magnification?

GREGG: The cell surfaces are stained, but otherwise no essential differences.

GROSS: Are there vacuoles in the prestalk cells?

GREGG: They become vacuolated when they differentiate into mature stalk cells.

GROSS: How much of the area of a section of such a cell would be vacuoles?

GREGG: What proportion?

GROSS: Let me phrase it this way. Are the prespore cells and prestalk cells about the same size?

GREGG: Prestalk cells are inclined to be a little larger in the slug.

GROSS: Now, is there a difference in the amount of vacuolated space in these two types of cells?

GREGG: The mature stalk cell is more vacuolated. It's a characteristic of stalk cells.

GROSS: Then it might simply reflect, not a difference in the number of antigens in the dry weight of the cells, but simply that there's a lot of empty space.

GREGG: Do you mean that the antigens are crowded out?

GROSS: Yes.

LOVETT: Is that actually true during the migrating stage when you get the same staining?

GREGG: No, the vacuolation does not occur until stalk cell differentiation.

TS'O: Have you tried mixing different antibodies together? If you have, do you see any different results?

GREGG: Takeuchi (1) used antisera made only to spores, and he gets exactly the same results as I found with antisera prepared from all three stages.

TILL: What would happen if you used antibody only to stalk?

GREGG: I think you could. In the first place, the stalk cells stain on their surfaces so there are some antigens there.

LOVETT: The only trouble with that is you'd have to be able to separate just the stalk. There is no stage at where there is only stalk.

TILL: Well, you could take prestalk cell area that doesn't stain very well.

GREGG: You can separate the stalks from the spores after fruiting body formation.

Figure 11 shows a culminating slime mold, *D. discoideum*. We notice that the intensity of the staining begins to diminish in the prespore area. This probably reflects the fact that the cells are about to form mature spores. I can't account for it otherwise. This is the prestalk area which, of course, remains relatively unstained.

Figure 12 shows a section of a mature spore mass of *D. discoideum*. The trouble with this sort of a preparation (cut at 5 microns) is that it's difficult to determine how many of the cells have been cut. The spores that are cut stain in their cytoplasm to a certain extent. Takeuchi believes they were stained in the cytoplasm, whereas certain cells appear to be stained only on the outside. He believed these particular cells were not sectioned. In general, however, the cytoplasmic staining seems to be reduced in the spores in my preparations. If you cut through a mass of spores, a considerable number of them must be cut. So my feeling is that the cytoplasmic antigen is relatively decreased and that, consequently, the staining is reduced in the spore cells.

SCHRAER: How large are the spores?

GREGG: They're about 5 microns. They're smaller than the vegetative amoebae or the cells in the later stages.

SCHRAER: Can you separate the spores and place them on a glass slide without sectioning them?

GREGG: Yes.

Now, as I mentioned a moment ago, obviously development of the slime molds depends on differentiation of these two types of cells

which are characterized by the loss of prestalk antigens in the prestalk cells and the synthesis of additional antigens in the prespores. To what this may be attributed is a little bit of a puzzle. However, we know from previous work that antigens are synthesized during the transition from the amoebae to the pseudoplasmodium. Also, antigens are lost during this transition. Some of these antigens which are lost may be the antigens from the prestalk cells, resulting in the loss of standing capacity in the prestalk area, whereas the additional antigens which are synthesized during the transition may be represented by the prespore antigens which are synthesized in the prespore cells. Additional antigens are lost and also synthesized in the transition from the slug to the mature spores.

Now, Sonneborn *et al.* (5) have shown that mucopolysaccharide begins to increase, beginning at about the late aggregation stage in *D. discoideum*, and reaches a peak during culmination. Perhaps the synthesis of prespore antigen is represented by a rise in mucopolysaccharide. However, this is speculation. Bonner *et al.* (6) have shown that such polysaccharides are confined to the prespore cells with very little polysaccharide staining in the prestalk cells. So, it's possible that some of the antigens we're dealing with are polysaccharides and that accounts for the particular staining we find with fluorescent antibody in the slug.

Now, there are some mutant forms of slime molds which appear to be inhibited in their ability to form prespore cells and prestalk cells. Filosa (3) isolated the mutant MV from *D. mucoroides*-11, which is characterized by the fact that it forms a relatively small slug. *D. mucoroides*-11 migrates long distances before forming the spore mass, whereas MV may migrate without forming a stalk or may migrate for a short distance and form a short stalk bearing a small spore mass. Thus MV appears to be inhibited in their migrating ability, and this may be tied up with the fact that they are inhibited in their ability to form prestalk cells and prespore cells. Figure 13 is a cross section of an MV slug, which in this instance has begun to produce a small stalk. Now, when we stain MV slugs with either MV serum or with wild type antiserum, we observe essentially the same staining pattern. Generally, the staining pattern is not as intensive as in the wild type and, on many occasions, the staining is spotty and gives a patchy appearance. There is no sharp delineation between the prestalk cells and the prespore cells. It appears that the normal complement of prestalk cells has not differentiated, at least by this stage. I might add that the antiserum produced by the MV will stain the wild type perfectly normally. So it appears that there are similar antigens in MV which can result in antiserum which will stain the wild type normally; but there apparently are not a sufficient number of antigens in the MV to enable it to stain intensively.

There is a mutant form of *D. discoideum* known as Fr-17 which is similar to MV in that it fails to develop normally (5). Usually it forms amorphous mounds of cells but under certain circumstances it forms an aberrant looking fruiting body, a stalk bearing a small spore mass. They have found this mutant, Fr-17, produces mucopolysaccharide in normal quantities but at a much earlier stage than the normal wild type. In other words, the production of mucopolysaccharide appears to be accelerated in the Fr-17. Incidentally, the mucopolysaccharide is antigenic, also (5). They've tested it with spore antiserum. So there's a possibility, since this polysaccharide(s) is antigenic, that we are dealing with polysaccharide as well as protein.

Now, Takeuchi (1) has reported that MV cells removed from the interphase stage, that is just prior to aggregation, still retain, what he terms, their ring-like staining. This means that they have more or less of a diffuse staining at interphase, whereas the wild type cell will have developed small granules which stain prominently. There appears to be a delay in MV at the interphase stage in the synthesis of these small granules. Whether this has anything to do with the polysaccharide synthesis we cannot say at the moment, but it appears that the differentiation of these cells into prestalk and prespores may be related in some way to the fact that they have delayed formation of these small granules which is normal to the wild type.

Most of the material that I've presented so far was a necessary prelude to the main point which I hope to make. I had to study normal development first in order to interpret the transection experiments which I shall discuss now.

Raper (7) performed an experiment with *D. discoideum* in which he transected prestalk cells and a portion of the prespore area and isolated the two fragments. If he allowed sufficient time to go by, each of these portions (the prespore area and the prestalk area) regulated to form a normal fruiting body. This means that each of these portions of the slime mold has the capacity to regulate. Consequently, each type of

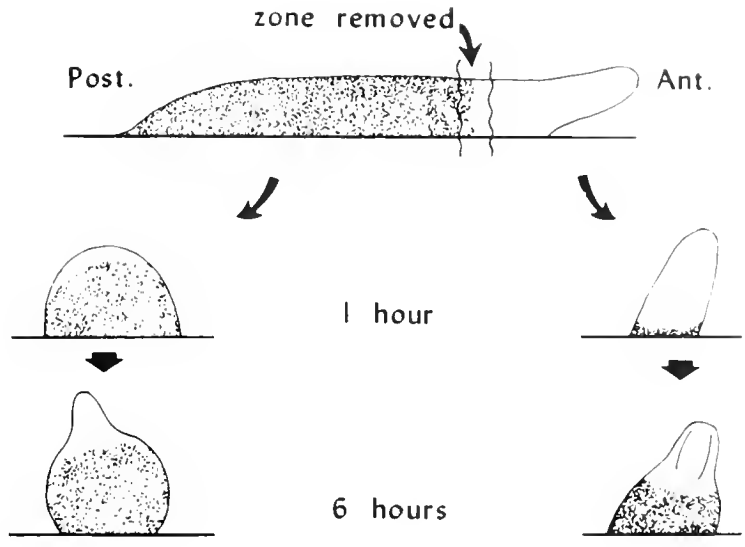
isolate can produce the missing cell type and produce a fruiting body of normal proportions.

Bonner *et al.* (6) following Raper's experiment in which they transacted the anterior tip and posterior prespore area in *D. discoideum* (Fig. 14). They discarded the center section in which the two types of cells were adjacent. I might add that the anterior tip is devoid of nonstarch polysaccharide staining (PAS) but the posterior tip is heavily stained. They allowed the isolated fragments to reorganize for one hour before fixing and staining again. They noted that in the isolated posterior prespore area a margin of cells had begun to lose staining which apparently marked the differentiation of prestalk cells. Later on the prestalk cells are more clearly established, and it appears that proportional development has been reestablished. In the isolated anterior tip the staining has begun in the lower region marking the beginning of the formation of prespore cells. By 6 hours prespore cell differentiation was well advanced. Thus, it appears that morphological reorganization or regulation of the slime mold occurs simultaneously with regulation of the biochemical entities.

We performed similar experiments with *D. mucoroides* and *D. discoideum* with the idea of staining the fragments with fluorescent antibody to determine the antigen patterns appearing during reorganization (Fig. 15). In *D. discoideum* transections we obtained about 2/3 of the anterior prestalk area and allowed it to reorganize for three hours before fixing it, running it through the sectioning process and staining it with antiserum. In *D. mucoroides* we isolated the anterior 2/3 of the prestalk area, trying to avoid the region we assumed to be close to the junction of the prestalk-prespore area. You cannot, of course, see the junction of the two types of cells in the living slime mold, unless they have been stained with some sort of vital dye beforehand. In other transections we cut as close as possible to the assumed position of the junction. Each of these fragments was allowed to reorganize for two hours before fixation.

A third type of transection was made which isolated the entire prestalk area and approximately an equal amount of prespore cells. This type of preparation was allowed to reorganize for two hours before fixation whereas the posterior prespore areas reorganized for 2 to 5 hours.

Figure 16 shows an isolated *D. mucoroides* anterior tip which was allowed to reorganize for about two hours. Now, prespore cells have



Flg. 14.

A diagram illustrating the experiment in which a partially differentiated migrating cell mass is bisected and each portion is examined by the PAS technique after one and 6 hours, respectively. Note that the anterior end of each fragment reversed its PAS staining properties; in one case from the light prestalk condition to the dark prespore condition and vice versa in the other. (Fig. 4, Bonner, Chiquoine and Kolderie, *J. Exp. Zool. 130*, 147, 1955; reproduced with permission of the Wistar Institute of Anatomy and Biology.)

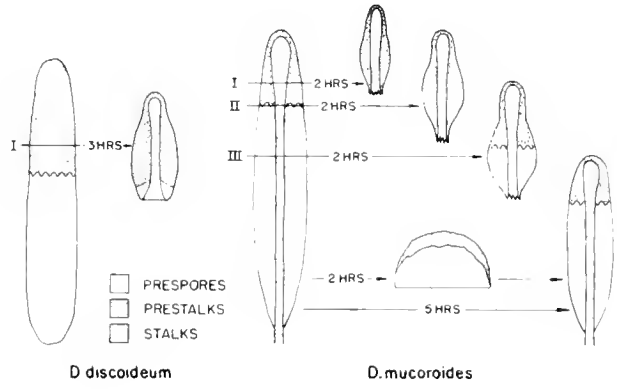


Fig. 15.

Diagram describing the transection of *D. discoideum* and *D. mucoroides* slugs and their developmental stages attained before fixation and staining with fluorescent antiserum. (Fig. 1, Gregg, Devel. Biol. 12, 377, 1965; reproduced with permission of Developmental Biology, published by Academic Press).

not differentiated in this particular preparation. It appears, although we did not make a detailed study of this, that the number of prespore cells that differentiate seems to depend upon the region in which the transection was made. The closer we get to the junction of the two types of

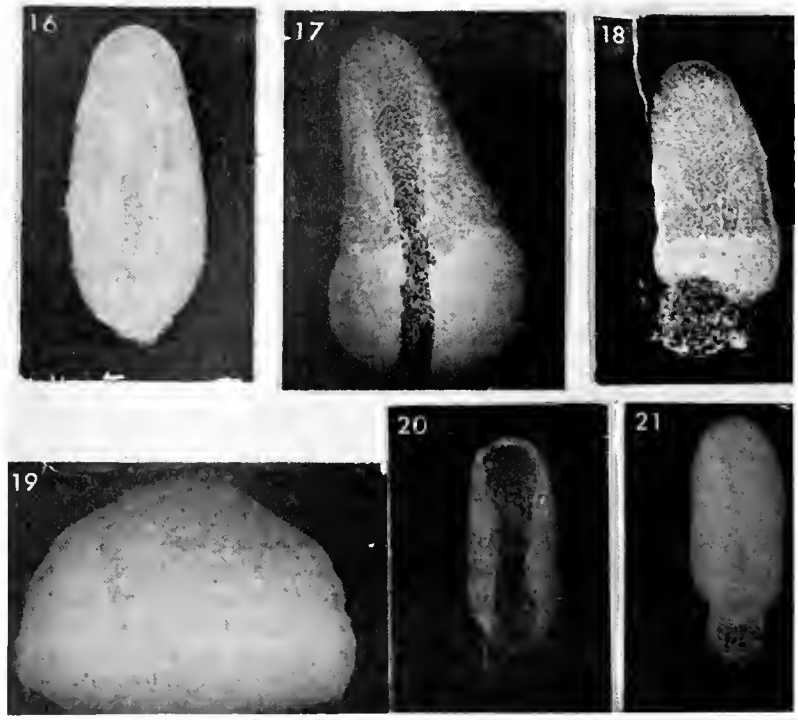


Plate II. Figures 16 through 21

Fig. 16. Transected *D. mucoroides* prestalk mass following a period of reorganization exhibiting an increased degree of staining in the prestalk and stalk cells as compared to a normal *D. mucoroides* slug (HFAS).

Fig. 17. An isolated *D. mucoroides* cell mass composed of approximately equal proportions of prestalk and prespore cells. Following reorganization the isolate was stained with HFAS. The prestalk cells did not stain cytoplasmically, apparently due to the presence of the prespore cells.

Fig. 18. Transected *D. discoideum* prestalk mass following a period of reorganization exhibiting an increased degree of cytoplasmic staining in the prestalk and stalk cells as compared to the same areas in a normal *D. discoideum* preculm (HFAS).

Fig. 19. Transected *D. mucoroides* prespore mass, following a period of reorganization, exhibiting intense staining in the prespore area and cell surfaces but lacking stain in the newly formed prestalk cells (HFAS).

Fig. 20. Transected *D. mucoroides* prestalk mass, following a period of reorganization, exposed to *D. mucoroides* vegetative myxamoebae absorbed HFAS. Fluorescent staining is completely negative.

Fig. 21. The identical histological described section in Fig. 20 but stained with HFAS. Staining exhibited by prestalk, stalk cells and cell surfaces.

(Fig. 18 from Gregg, *Devel. Biol. 12*, 377, 1965; reproduced with permission of Academic Press.)

cells the more we were apt to obtain prespore cell differentiation. Now, if we allowed such fragments as these to complete culmination and form fruiting bodies, we observed small numbers of spore cells, sometimes undifferentiated cells, and, of course, stalk cells. Thus, the isolates produce prespore cells but the abundance depends to a certain extent upon the proximity of the transition to the prestalk-prespore junction.

The most striking thing about the reorganized anterior tip was the tremendous increase in the amount of antigen that reappeared. Generally, mature stalk cells do not contain such a tremendous amount of antigen as this. The prestalk cells in many of the preparations were completely uniformly stained. So apparently an antigen reappears during the reorganization process. The fact that it appears much more intensely in the stalk cells may simply result from a difference in the geometry of the cells relative to the prestalk cells. The antigen is probably resynthesized in the prestalk cells which, of course, form the stalk cells during the reorganization process.

Figure 17 shows a fragment that was isolated, composed of about the same number of prespore cells and prestalk cells. Now, we find that these prestalk cells in the presence of the prespore cells do not synthesize the antigen. It

appears that the presence of the prespore cells in some way inhibits the synthesis of this antigen that appears in the isolated prestalk cells.

Figure 18 shows an isolated *D. discoideum* prestalk area which was allowed to reorganize for a couple of hours. Again you find a reappearance of the antigen in the prestalk cells and in the stalk cells. In *D. discoideum* prespore cells seem to differentiate more readily. I believe that these are newly differentiated prespore cells and not prespore cells which were accidentally removed at the time of the transection. So, I believe that proportional reorganization was initiated in this preparation.

TS'O: Excuse me, one thing is not very clear to me. Is the rearrangement or reorganization involved with synthesizing new types of cells or transformation of old types to new types?

GREGG: In an isolated prestalk area, in order for them to regain their proportionality, they must differentiate new prespore cells from existing prestalk cells.

TS'O: There is new synthesis going on, too, isn't there? Don't you get new cells?

GREGG: No, there's no increase in cell number.

TS'O: Then the transected one would be smaller in size?

GREGG: Oh yes, it would be smaller. The size depends upon the total number of cells isolated.

Figure 19 shows a reorganizing *D. mucoroides* posterior prespore area. Now if we fixed and stained this immediately following transaction, the entire area would be stained uniformly. After two hours of reorganization the cells at the anterior or uppermost part are beginning to lose their stain or cytoplasmic antigen. Evidently, they are forming prestalk cells in order that they can form a stalk and consequently a fruiting body. If we allow reorganization to go on for about five hours, the normal slug shape is regained and the normal proportion of cells is restored.

In order for a slime mold prestalk isolate to regain its proportionality it must differentiate a certain number of prespore cells. The antigen that reappears must be a necessary entity in the formation of new prespore cells. The preparation in Fig. 20 was stained with antiserum which was absorbed with vegetative amoebae from cultures of about 17 hours of age. We were interested in determining whether nor not the new antigen which reappeared was the same antigen which was present in the cells of an earlier age. If it was present in younger cells it probably was necessary in the initial differentiation of prespore cells and prespore antigens. However, the absorbed serum produced no staining whatsoever in this preparation. Now, had this preparation contained prespore cells, the prespore cells would be stained to a certain degree. If the antiserum is absorbed with vegetative amoebae, the prespore staining is not removed. It removes all the staining in the prestalk cells, however.

Figure 21 shows exactly the same section with the exception that it has been stained with the non-absorbed serum simply to show that the antigen had been synthesized in this particular preparation. Now, it was of interest to us that this antigen appeared throughout the entire prestalk area. If it is necessary for the slime mold to produce prespore cells to regain their proportionality and if this antigen is necessary in the reorganization, it's strange that it was not confined only to a certain number of prestalk cells which would be likely to form prespore cells in the lower area. Thus, it appears that the isolated prestalk cells cannot immediately integrate their size with the necessity to differentiate a particular number of prespore cells. This is based on the assumption that this antigen is an antigen necessary in the differentiation of prespore cells.

How does this proportionality arise? Well, I suggest that proportionality arises from the differentiation of prespore cells; and as a result of the differentiation of prespore cells, there is an interaction between the two types of cells which results in an equilibrium. Consequently, in some way, the differentiation of the missing cell types is limited such that the cells are not over-produced. Now, the same idea may be applied to the isolated prespore areas. Their "task", of course, is to produce new prestalk cells. As prestalk cells are produced, again I suggest that there is an interaction between the two cell types which results in a control of cell differentiation and eventually results in proportionality being established.

FERGUS: Would you care to comment on why those prespore amoebae could not increase in number in your transection techniques?

GREGG: Well, I could not state categorically that cell division does not occur. However, attempts have been made to find whether or not the cells increase in number during the normal development, not particularly in transection. There has been no finding which definitely established that there was a tremendous increase in

cells, if any at all. You see occasional mitotic figures but apparently there is no significant increase in cell number.

FERGUS: Most of this has occurred prior to the formation of the slug?

GREGG: Yes. As a matter of fact, division apparently does not occur after aggregation. Furthermore they utilize only endogenous foodstuff during morphogenesis.

FERGUS: You were working here with no external source of food?

GREGG: Yes. They feed upon bacteria during vegetative amoebae stage; and once they aggregate, they can carry out this whole developmental process in the complete absence of foodstuffs.

PERSON: Is this a buffered medium?

GREGG: This is on agar; they're buffered at about 6.2. Complete morphogenesis occurs on this medium.

GROSS: This certainly ought to dispel one of the pet ideas of a number of embryologists that is still quoted very widely: namely, that differentiation and dedifferentiation are processes that are intimately linked with cell division. Dedifferentiation itself is not demonstrated.

GREGG: This appears to be a form of dedifferentiation.

DEERING: You have type *a* (prespore) changing to type *b* (prestalk) or type *b* changing to type *a*, either way?

GREGG: Yes, and this occurs in the absence of an increase in the mass of cells.

KAHN: I think that this is a very interesting point. I must confess that I've always felt that cell differentiation (morphogenesis) in these organisms was independent of cell division, but I'm beginning to think, in terms of this experiment, that this point should be tested. After all, these amoebae do have a fair amount of endogenous reserve. For example, if spores are placed in a suitable environment they will germinate and may complete the life cycle a second time in the absence of exogenous nutrient.

GROSS: Are you implying that there is cell replication?

KAHN: I'm implying that it's possible.

GROSS: However, in order to have something that approximates the old hypothesis that the decision is made at mitosis, you'd have to, at least, double the number of cells. I should think that could easily be seen.

GREGG: I don't believe that cell division is necessary, because you can cause fruiting body formation from small quantities of cells. As a matter of fact, fruiting bodies have been obtained from as low as 7 cells.

GROSS: Is that an adult fruiting body?

GREGG: Yes. Obviously cell division isn't necessary here although it's true no one has examined a larger isolated anterior tip.

KAHN: I think there's a point worth stressing about regulation (developmental) in cellular slime mold development. For example, Bonner has shown that normal development can occur in aggregates containing fewer than 100 cells as well as in aggregates containing many thousands of cells. In the slime mold, *Acytostelium*, even a single amoeba may show developmental regulation. In this case the cell gives rise to a structure composed of a single spore perched on an acellular stalk.

GROSS: At any rate, I think it's helpful to the state of the problem so as to have these things discussed more widely than they are. Most people don't know about this particular point. It's such a clear case of a switch in the choice that the cell makes about what it's going to do, a switch that can be produced externally without any massive cell replication.

GREGG: It's one of the most striking things about cellular slime molds.

GROSS: If it's true that these cells are really not replicating, then all of this may happen during interphase. This immediately rules out any proposal about sequential nature of transcription in microorganismal cells like this. If these cells decide to go back and become another cell type, they're really making different antigens which means different genes are being transcribed. On the basis of the biology of this system it would very unlikely that they would go back and transcribe the whole genome in order.

LOVETT: Could they go back and start in the middle?

GROSS: It seems to me it doesn't argue against the sequential transcription so much as it does that it's obligatory that it starts at one end and can't do anything until it reaches the other end, and then starts over again.

TS'O: That model you have in mind, Paul, must be a linear one and not a circular one.

GROSS: Yes.

GREGG: It's hard to say whether they start at the beginning or in the middle. If you examine a cell mass, you see what appears to be a sort of a background fluorescence, and then when you get prespore differentiation in the late aggregate, you see spots of prespore antigen. The antigen that reappears in the isolated one appears to be this background antigen which is present

in the vegetative amoebae and easily observed in the early aggregate. So, the background antigen appears first; and this is followed up by the synthesis of the prespore antigen. I doubt if the cells would synthesize prespore antigen in the absence of this background antigen. I think the isolated prestalk cells try to establish conditions as they were in the early stages of normal development as a prelude to differentiating into prespore cells.

EPEL: Relating to Paul's point, maybe this is a difference between microorganisms and metazoans; if this *is* a microorganism.

GREGG: It is claimed taxonomically by both the botanists and zoologists.

GROSS: Are you referring to the capacity for dedifferentiation?

EPEL: Yes. Is this like a bacterial spore or protozoan, which forms a spore under certain environmental conditions?

DEERING: Can you take these things after you've cut them once, have them change, and cut them again and have them change back again?

GREGG: If you don't wait too long, I should think you could.

DEERING: In other words, you can change *b* to *a* and then back to *b*? I wonder how long you could keep this up?

GREGG: Probably until you get down to a very few cells.

KAHN: A good deal of our discussion has centered around cell metaplasia, the ability of a cell to exist in different states. Recently, Dr. Lindsay Olive (Columbia University) described an amoeboid microorganism that is capable of assuming amoeboid, flagellate, cyst or spore form. It would be very interesting to know whether this organism can make these transformations in the absence of cell division.

MASSARO: Isn't it possible, let us say, that certain of these cells in a particular area are like reserve cells, not being particularly committed at any one time to any one tissue; and these cells perform the reorganization?

LOVETT: I don't think it's necessary to assume that the cells in layer X are identical to the cells in layer Y; but cells in layer X may be dedifferentiated, undifferentiated, or less differentiated cells which are in reserve to be committed to the reorganization or formation of the structure.

GREGG: You mean this is the case just in the event someone comes along and cuts one in half?

LOVETT: Certainly. I respect the potential of these cells.

GREGG: With regard to your remarks I can only say this: in *D. mucoroides* we've thought about this to a certain extent. Prestalk cells have to be continually replaced by the prespore cells as the slug crawls along because the prestalk cells are continually forming stalk. So in order to keep the proportions of these cells constant it has to keep replenishing the prestalk cells. Now, there appears to be a gradient of differentiation between these two regions. In other words, the further anterior you cut, the more apt you are to get fewer spore cells and more undifferentiated cells following a reorganization period. The closer you cut to the prestalk-prespore junction the greater number of cells you get which have just crossed the border into the prestalk area. Consequently, it's much more likely that they can dedifferentiate to form prespore cells. I don't know whether this answers your question about reserve cells or not.

DEERING: Can we really eliminate the possibility that there is a third type of cell that can go either way and that this is what always leads to appearance of new types?

GROSS: I think you can.

GREGG: I suppose it would be possible.

DEERING: In other words, you can't really eliminate that possibility. I think it's important.

GROSS: But the requirement is that if you had such a population of cells, they would have to be uniformly distributed throughout the slime.

MASSARO: Why is it necessary to have a uniform distribution?

GROSS: Because you get regulation wherever you cut. If they were restricted to one end, then you wouldn't get regulation at the other end.

LOVETT: However, don't you get varying degrees of reorganization depending on where you cut?

GREGG: Yes, fruiting body formation invariably occurs although the proportions of the two types of cells composing it depends upon where you cut and the amount of time the prestalk mass requires to reorganize.

LOVETT: All pieces of the slug cut at the proper stage will eventually reorganize and regulate the proper proportion between prestalk and prespore cells?

GREGG: Presumably if they're allowed to migrate long enough they reestablish their proportions. I think one of the reasons that you did not see prespore cells immediately in the anterior tips that I showed you was due to the fact that the anterior tips rush right into fruiting;

and I think they rush into fruiting so fast that they do not have time to form prespore cells proportionally in all instances.

TILL: Will the spores that you get from these transections give a normal organism?

GREGG: Oh, I'm sure they would.

MASSARO: To go back for a minute. What did you mean by uniform distribution? Do you mean X number of cells surrounded by Y number of undifferentiated ones?

GROSS: Yes. If there's another type of cell that is neither prestalk nor prespore, they have to be somewhere in the slug. Now, when you cut, you can cut any part of it, and in principle, you can get back the whole thing.

MASSARO: You could have the third type of cell anywhere.

GROSS: That's the point; they are anywhere. They're a population of finite size. Now, as you reduce the sizes of pieces you cut, the fraction of the uncommitted cells relative to those cells that have already differentiated is going to change depending on where you cut. If you cut in the anterior end, you're going to have a large number of prestalk cells and a very small number of prespore cells; and you still have a small number of undifferentiated cells. There's no replication, so you've got a large number of prestalk cells that can't go anywhere and no prespore cells; now, the small number of uncommitted cells must, in that instance, all differentiate to form prespore cells. Suppose you don't have enough. It seems to me that as the piece gets smaller, like 7 cells, you're not going to have enough of those relatively uncommitted cells.

MASSARO: Well, maybe these cells have only a certain degree of noncommittedness. Maybe we're looking at the noncommitted cells too harshly and saying we have a cell here which is definitely noncommitted. Maybe certain prestalk cells are less committed than other prestalk cells.

GROSS: This is an argument that extends far beyond the slime molds. It's one that has plagued embryologists for many years.

KAHN: Jim, did you look at *Polysphondylium* at all?

GREGG: No, I didn't.

KAHN: Well, this might be worthy of mention along these lines. If you do the same sorts of things that Gregg has done with fluorescent technique with various histochemical techniques, you do get a differential staining between the presumptive stalk and the presumptive spore areas. This is true in *Dictyostelium discoideum*, also.

GREGG: The presumptive stalk region is a very small area.

KAHN: I was going to get to that. The interesting thing about *Polysphondylium* is that you don't see these differences until very late; so, in effect, the whole mass is uncommitted until the very last moment.

GREGG: You can differentiate between the types of cells in a number of ways: PAS staining, vital stains, antibodies.

PERSON: Is there a vital stain that can differentiate between the two types of cells so that you could keep an individual cell alive and look at it?

GREGG: Yes. Bonner's used stains such as Nile blue sulfate, neutral red and Bismarck brown.

GRUN: Do they all produce a darker staining in the nonstalk area and a lighter staining in the stalk area?

GREGG: I believe the staining is more intense in the anterior end with most of these stains.

ACKNOWLEDGEMENTS

The meticulous histological preparations which were made by Mrs. Doris Gennaro during the course of this investigation are gratefully acknowledged by the author.

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CONTROL OF ENZYME ACTIVITIES IN *D. DISCOIDEUM* DURING DEVELOPMENT

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I believe the usual concept of morphogenesis includes a visible change in the form or structure of an organism. This implies a gradual accumulation or redistribution of structural material, such as connective tissue, bone or cell wall polysaccharides, for example. This, in turn, implies alterations in the activity of enzymes responsible for the synthesis of these materials. A number of possible mechanisms for changing the activity of an enzyme appear in Fig. 1. This figure summarizes various ways in which the product characteristic of a particular differentiated cell might be made to accumulate during development. The rate of product accumulation could be enhanced by an increased level of the enzyme, substrate, activator or RNA template used in the synthesis of the enzyme. The accumulation of any of these types of molecules, of course, implies nothing with respect to the mechanism. The three possibilities are a) an increased rate of synthesis, b) a decreased rate of destruction or c) the activation of a preformed inactive form of the molecule. Thus, for each of the mechanisms listed in the figure the problem is simply pushed back to another level of analysis.

Although our present state of knowledge allows the discussion of these three possibilities only with respect to enzyme levels as indicated in the figure, levels of the other types of molecules would be altered, also, by similar mechanisms. Finally, it must be kept in mind that an observed increase in level of any of these factors would be critical to the formation of a product of differentiation only if it were already limiting the process in the cell. Such information is exceedingly difficult to obtain. Changing levels of an enzyme or a substrate may only be correlated with, and an indirect result of, the morphogenetic process observed and may be due to causes quite unrelated to our naive and prejudiced interpretation. The fact that DNA and RNA play an important part, at some point, in controlling the details of cellular differentiation need not be documented. The question concerns the *time* at which their action is necessary relative to the unfolding of a particular developmental process.

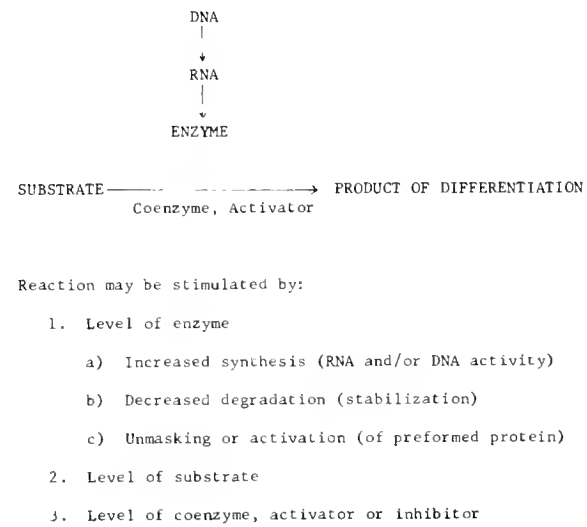

Fig. 1.

From Wright, Barbara E.: Control of Carbohydrate Synthesis in the Slime Mold. In *Developmental and Metabolic Control Mechanisms and Neoplasia* (A Collection of Papers Presented at the Nineteenth Annual Symposium on Fundamental Cancer Research, 1965), p. 297. Baltimore, The Williams and Wilkins Company, 1965.

Activity at the enzyme and substrate level must necessarily be correlated in time with the accumulation of the product characteristic of the differentiated cell. This need not be true, of course, for the nucleic acid template responsible for the presence of these enzymes. In fact, recent studies of Brown in the amphibian, Gross in the sea urchin and Sussman in the slime mold indicate that certain stages of differentiation do not, in fact, depend upon the concurrent formation of messenger RNA. This situation brings renewed interest to other types of control, such as the activation of preformed mRNA, enzyme accumulation through lack of degradation, enzyme relocation within the cell, the availability of a substrate or an enzyme activator, etc. Regardless of the relative importance of nucleic acid control during a particular process of differentiation, the cellular environment of the enzymes involved is critical, of course, in determining the nature and extent of their activity. In other words, since the action of an enzyme is entirely dependent upon levels of specific substrates, activators, inhibitors and the like, knowledge of these variables in the intact cell is essential in attempts to evaluate the significance either of a constant or a changing enzyme level to a reaction important to development.

Let me illustrate this point by mentioning just two examples in the slime mold. The morphogenesis of this microorganism depends, in part, upon the breakdown of endogenous protein and its eventual conversion to carbohydrate. As protein degradation intensifies during development, the intracellular concentration of glutamate increases an order of magnitude. Oxidation of this amino acid and its entry into the Kreb's cycle is a necessary step in its utilization for carbohydrate synthesis. The enzyme responsible for this oxidation, glutamic dehydrogenase, is very stable in extracts prepared throughout development. Although the concentration of this enzyme does not change, its activity when measured *in vivo* using radioactive glutamate increases 7-fold during development. The dehydrogenase was purified and its affinity for glutamate was determined; knowing the effect of substrate concentration on the rate of this reaction, it was shown that the accumulation of glutamate *in vivo* could fully account for the enhanced rate of the reaction in differentiating cells. Thus, data at the enzyme level was insufficient in interpreting the *in vivo* activity of this enzyme during development (1).

The slime mold offers another example of an enzyme which does increase in concentration during development (some 6-fold), yet this change is not reflected in its activity *in vivo*. Dr. Gezelius has studied an alkaline phosphatase, highly specific for 5'-AMP, which reaches a maximum *concentration* at the end of differentiation. However, inhibition of the enzyme by increasing levels of inorganic phosphate *in vivo* results in maximum *activity* of the enzyme not at the end but in the middle of differentiation (2). Thus, observed alterations in the concentration of an enzyme may not bear a direct relationship to its actual activity in the differentiating cell. This is probably the rule rather than the exception.

Enzymes are usually measured under conditions of pH, inonic strength, substrate concentration, co-enzyme, activator or inhibitor concentrations, which do not reflect the conditions in the differentiating cell. Much more data are needed in which enzyme activities are measured both *in vivo* and *in vitro* and in which levels of relevant substrates, co-enzymes and activators are determined *in vivo* at various stages of development. All these data, taken together, may then give a consistent picture of the activity of an enzyme in differentiating cells.

To facilitate the following discussion, I will very briefly summarize the life cycle of *D. discoideum* (Fig. 2). Upon starvation, the cellular slime mold passes from the vegetative stage, during which it exists as a homogeneous population of myxamoebae lacking a cell wall, through an aggregation process to become a differentiated multicellular organism. Successive stages which I will refer to are known as aggregation, pseudoplasmodium, preculmination, culmination and sorocarp or fruiting body. In the terminal stages of development the cells are ensheathed in a cell wall composed of a cellulose-glycogen polysaccharide complex, the synthesis of which will be the subject of a good portion of my presentation. All of the experiments I will talk about were done with cells which were starving on 2% agar throughout the differentiation cycle.

Figure 3 summarizes the general area of metabolism with which we will be concerned. Endogenous material, such as protein, is degraded and gluconeogenesis begins. Hexose phosphates are formed and glucose-1-phosphate together with UTP unite to form uridine diphosphoglucose (UDPG), an essential precursor to cell wall material. Phosphoglucomutase, interconverting G-1-P and G-6-P, is very active throughout development, as is pyro-

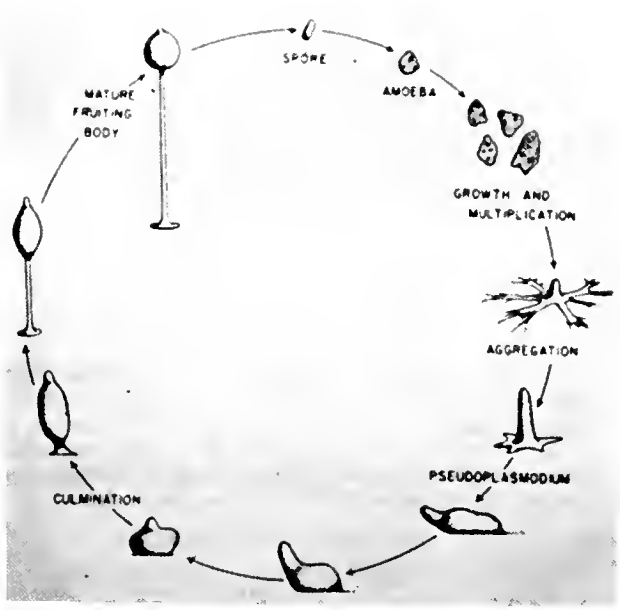

Fig. 2.

phosphatase. This would tend to aid the accumulation of UDPG by removing pyrophosphate. Neither of these enzymes changes strikingly during development, but UDPG synthetase does increase about threefold at culmination.

A number of precursors of cell wall material increase and then decrease prior to sorocarp construction. Figure 4 shows data obtained by Mr. Beers in our laboratory on glucose-6-phosphate accumulation in a number of stage studies. As you can see, there is a good deal of variation from one stage to another, but in general, glucose-6-phosphate reaches a peak at culmination.

Figure 5 is a schematic summary of the accumulation pattern of a number of polysaccharide precursors and of some end products of differentiation. Gluscose, glucose-6-phosphate, glucose-1-phosphate and UDPG increase and decrease in the cells during development as cellulose, mucopolysaccharides, trehalose and an alpha-1, 4 polymer, which I will discuss, accumulate. Since cell wall construction occurs only at the terminal stages of development, it represents an excellent index of differentiation. We wanted to know exactly what conditions in the starving, differentiating cells set the stage for cell wall accumulation. This work was done in collaboration with Carole Ward and Donna Dahlberg.

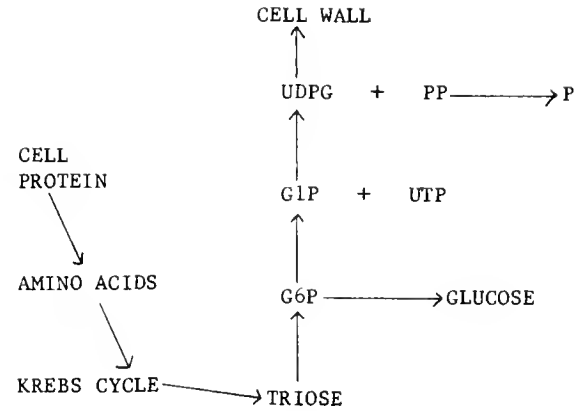

Fig. 3.

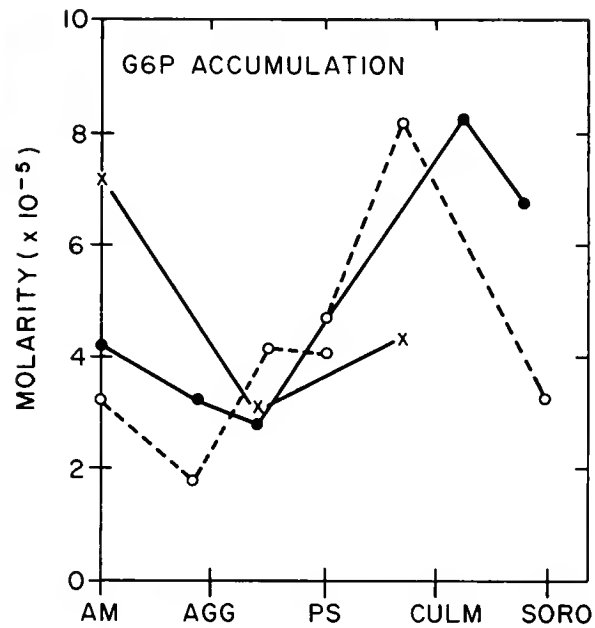

Fig. 4.

From Wright, Barbara E.: Control of Carbohydrate Synthesis in the Slime Mold. In *Developmental and Metabolic Control Mechanisms and Neoplasia* (A Collection of Papers Presented at the Nineteenth Annual Symposium on Fundamental Cancer Research, 1965), p. 301. Baltimore, The Williams and Wilkins Company, 1965.

In order to study cell wall synthesis *in vitro*, husk preparations of culminating or terminal stage cells were made by passing the cells through a French pressure cell and then washing extensively in tris-EDTA buffer. These cells were then incubated with radioactive UDPG,

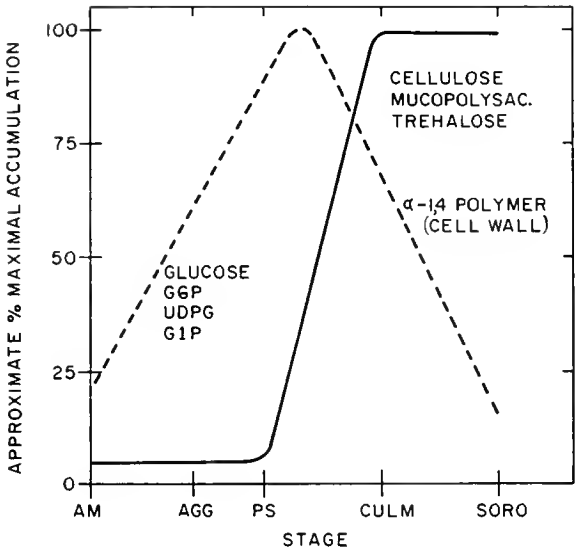

Fig. 5.

TABLE I

Stability of Enzymatic Product [a]

EXPERIMENT		
I	Boiled 10 min., 5% NaOH	900
	Boiled 20 hrs., 1% NaOH	947
	Boiled 20 hrs., 1% NaOH + 2 hrs., 2.5 N H_2SO_4	55
II	Boiled 10 min., water, NO NaOH	1,584
	Boiled 10 min., 30% NaOH	1,494

[a] From Wright, Barbara E.: Control of Carbohydrate Synthesis in the Slime Mold. In *Developmental and Metabolic Control Mechanisms and Neoplasia* (A Collection of Papers Presented at the Nineteenth Annual Symposium on Fundamental Cancer Research, 1965), p. 303. Baltimore, The Williams and Wilkins Company, 1965.

TABLE II

Substrate Specificity

EXPERIMENT	SUBSTRATE	μMOLES	μMOLES GLUCOSE INCORP. (x 10^3)
I	UDPG	1.0	39.0
	G6P	1.0	0
	GLUCOSE	1.0	0
	UDP-GAL	1.0	0
II	UDPG	0.2	5.0
	GDPG	0.2	0.5
	ADPG	0.2	0.2

the reaction stopped by boiling, carrier cellulose added and the material washed repeatedly, boiled in alkali, wash some more and finally counted in a scintillation counter.

Table I indicates the alkaline and acid stability of the radioactive, alkali-insoluble product. Gezelius and Ranby isolated comparable material from *D. discoideum*, and the most rigorous treatment in their purification was twenty hours at 100°C in 1% alkali. They studied this material very carefully by x-ray diffraction and other types of analyses and concluded that it was an amorphous form of cellulose (3). They found only glucose on acid hydrolysis. In confirmation of this, we found only radioactive glucose on acid hydrolysis of our radioactive cell wall material. A substrate specificity study revealed that UDPG was by far the preferred substrate (Table II). GDPG, which has been recently shown by Hassid's group to be a precursor to cellulose synthesis in plants (4), was only about 1/10 as active.

We were able to carry the purification of cell wall material one step further than Gezelius and Ranby, and separate it into two fractions, *A* and *B*, by solution in a cuprammonium hydroxide solution known as Schweizer's reagent (Table III). We will be talking now just about fraction *A* and soluble fraction *B*, not insoluble fraction *B*. After solubilization in the cupric ammonium hydroxide solution, fraction *A* precipitated out on neutralization and the addition of water. This material is not water soluble. Fraction *B* precipitated out from the supernatant following the addition of ethanol. Chemical, enzymatic and chromatographic analyses of the radioactive and nonradioactive fraction *A* and fraction *B* have identified the latter as an alpha-D-1, 4-linked polymer and fraction *A* as cellulose. Some of the enzymatic analyses are summarized in Table IV. Oyster glycogen was used as a control. The expected limit dextrin was made from nonradioactive, insoluble fraction *B* by phosphorylase treatment. Complete degradation was achieved by further attack of amylo-1, 6 glucosidase. Analysis of radioactive material revealed that most of the radioactivity is incorporated into fraction *B* and that fraction *A* is contaminated with the alpha-D-1, 4-linked polymer. Thus our studies have led to the conclusion that the alkali-insoluble cell wall ma-

TABLE III

Fractionation of Radioactive Cell Wall Material with Schweizer's Reagent

Exp.	Original	Undissolved residue	Total cpm Frac. A	Frac. B (soluble)	Frac. B (insoluble)	% Total recovery
I	2,140	132	400	1,267	-	85
II	138,000	200	2,770	70,000	10,000	60
III	128,900	-	19,790	68,620	-	68
IV	130,345	8,310	8,074	77,850	3,770	82

TABLE IV

Hydrolysis of Fractions A and B

Sample	Enzyme Treatment α-1,4-phosphorylase	Enzyme Treatment amylo-1,6-glucosidase	% Hydrolysis
Oyster glycogen	present	absent	38
Oyster glycogen	present	present	100
Non-radioactive Frac. B	present	absent	41
Non-radioactive Frac. B	present	present	90-100
Radioactive Frac. B	present	absent	100
Radioactive Frac. A	present	absent	60-80

terial is composed not of cellulose only, but rather of a 50-50 mixture of cellulose and glycogen polysaccharides in intimate association. During synthesis of this material *in vitro* most of the radioactive glucose in UDPG-^{14}C is incorporated into the glycogen polymer (5).

Let us now turn to some properties of the enzyme system catalyzing the synthesis of cell wall material from UDPG (Fig. 6). We determined the activity of the enzyme as a function of UDPG concentration. The UDPG concentration does not change significantly during polysaccharide synthesis *in vitro* in the presence of a well-washed particulate preparation. Therefore it seems justified to consider 1.3×10^{-3} to be the approximate K_m for UDPG in the synthesis of cell wall material. During differentiation in the slime mold the intracellular concentration of UDPG is well below 10^{-3} *M* except in culminating cells which are rapidly accumulating cell wall polysaccharides. Assuming that the UDPG values approximate the concentration available to the enzyme *in vivo*, it would appear that UDPG is one limiting factor to the initiation of cell wall synthesis in the differentiating cell. Conversely,

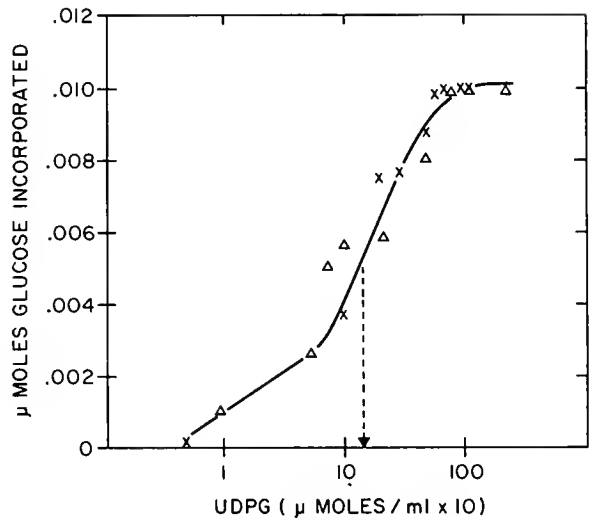

Fig. 6.

From Wright, Barbara E.: Control of Carbohydrate Synthesis In the Slime Mold. In *Developmental and Metabolic Control Mechanisms and Neoplasia* (A Collection of Papers Presented at the Nineteenth Annual Symposium on Fundamental Cancer Research, 1965), p. 311. Baltimore, The Williams and Wilkins Company, 1965.

the depletion of UDPG, which occurs very rapidly during sorocarp construction, would of course be a determining factor in the termination of polysaccharide synthesis.

Both glucose-6-phosphate and magnesium stimulate cell wall polysaccharide synthesis *in vitro* (Table V). G-6-P is known to lower the K_m for UDPG in a number of other systems in which glycogen synthesis has been studied. If one adds magnesium extracellularly in the 2% agar in which the slime mold is differentiating, it increases the rate of overall differentiation

TABLE V

Stimulation by G6P and Mg^{+2}

Additions	c.p.m. in alkali-insoluble material	
	Day 1	Day 2
None	261	485
G6P (2×10^{-3} M)	973	2,158
$MgCl_2$ (1×10^{-3} M)	309	603
G6P + $MgCl_2$	1,508	2,317

TABLE VI

Stimulation of Polymer Synthesis by Trehalose[a]

Additions	c.p.m.
---	518
G6P (10^{-3} M)	730
Trehalose (10^{-3} M)	471
G6P + Trehalose	910

[a] From Wright, Barbara, E.: Control of Carbohydrate Synthesis in the Slime Mold. In *Developmental and Metabolic Control Mechanisms and Neoplasia* (A Collection of Papers Presented at the Nineteenth Annual Symposium on Fundamental Cancer Research, 1965), p. 312. Baltimore, The Williams and Wilkins Company, 1965.

considerably. It is possible, therefore, that magnesium is limiting cell wall synthesis during development. In the experiment shown in Table V the enzyme was prepared on day 1 and assayed immediately in the presence of UDPG-^{14}C; when the enzyme was aged for a day and assayed on day 2, it had become activated, but the requirements remained comparable. I'll discuss activation later.

Table VI shows that trehalose stimulates cell wall synthesis, but only in the presence of G-6-P. We don't understand the mechanism for trehalose stimulation, but I want to make the point that trehalose, according to Filosa, accumulates very late in development during sorocarp construction, so that trehalose would probably be a limiting factor at culmination for cell wall synthesis.

Table VII shows a complex relationship between UDPG concentration and glucose-6-phosphate concentration in their effect on cell wall synthesis. It can be seen that G-6-P only stimulates cell wall synthesis at a low level of UDPG, but not at a high level. Therefore, their effects are interdependent. Although it isn't shown in this table, the concentration of G-6-P which maximally stimulates is about 10^{-3} *M* and in the intact cell it never reaches a level higher than 10^{-4} *M*. Glucose-6-phosphate would, therefore, presumably be limiting in the cell for cell wall synthesis. For unknown reasons intracellular UDPG levels vary significantly from one stage study to another. Although the maximum concentration is always at culmination, the level throughout differentiation in a particular stage study may be unusually high or unusually low. Thus, G-6-P could serve as a buffering agent, exerting strong stimulation at low UDPG levels and less stimulation in cells which are not as limited with respect to their UDPG levels.

It is apparent from these and many other studies that the existence *in vivo* of many limiting factors for the synthesis of materials important to differentiation may be the rule rather than the exception. It is known that even in fully differentiated cells enzymes are usually operating far below their potential activity due to substrate limitation. Cells undergoing differentiation are frequently dependent entirely upon endogenous metabolism and have very limited resources from which to obtain the necessary energy and building blocks for the many synthetic processes required in morphogenesis. If, in fact, it is true that multiple limiting factors

TABLE VII

Interdependence of G-6-P and UDPG

Final molarity		μMoles incorporated
G-6-P	UDPG	($\times 10^3$)
None	10^{-4}	0.29
10^{-3}	10^{-4}	1.10
None	10^{-2}	44.0
10^{-3}	10^{-2}	40.5

are always associated with development, one might well inquire into the possible advantage this situation could bring to the differentiating cell. I would like to suggest that the least precarious approach for a differentiating cell actually may reside in its dependence upon a complex interplay of many limiting factors. In this way, unusual deficiencies or abundances in the cell or the cell's environment need not necessarily upset the process of differentiation.

Let me elaborate on this concept briefly, using some recent work with hexokinase (Fig. 7). In the figure on the left the reciprocal of the glucose concentration is expressed on the abscissa and the inverse of the velocity of the reaction on the ordinate. Velocity is seen to increase with increasing levels of ATP from 0.1 to 1.2 millimolar. In other words, the reaction is stimulated by ATP in the presence of limiting levels of glucose. Similarly in the right part of this figure increasing levels of glucose stimulate the reaction in the presence of limiting levels of ATP. When both substrates are limiting, increasing the concentration of either increases the rate of the reaction (6). Thus, if this situation prevailed in the cell, increasing levels of either ATP or glucose could increase the level of glucose-6-phosphate. If, on the other hand, either substrate were in excess, G-6-P accumulation would depend upon the concentration of the other substrate. In this sense the system would be less flexible than if both substrates limited. Such flexibility may be very important to the stability and reproducibility of differentiation.

Let us now turn from complications at the substrate level to even greater complications at the enzyme level. We have said nothing as yet concerning the enzyme activity during the earlier stages of development. Figure 8 shows one of our earlier experiments in which we compared enzyme activity at various stages of development with the amount of alkali-insoluble material present. The stages of development are amoeba (A), aggregation (agg), preculmination (PC), a combination of culmination and fruit (CF) and fruit (F). Aliquots of cells were harvested at various stages of development and the percent dry weight of the cell wall material determined. This is indicated on the left ordinate. At each stage, also, a particulate enzyme of cell wall or cell membrane preparations was prepared in tris buffer and 10^{-4} *M* EDTA and was incubated with radioactive UDPG. The alkali-insoluble radioactive product was isolated, counted and related to the dry weight of the sample. The specific enzyme activity was thus determined,

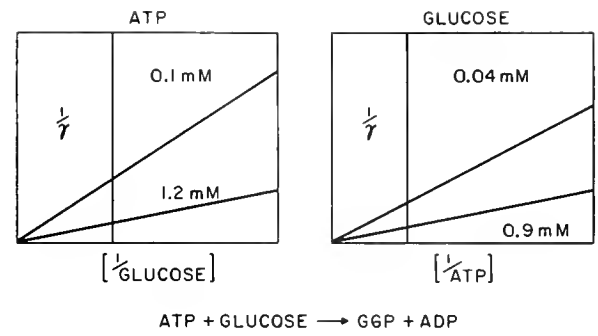

Fig. 7.

(Fig. 3, From Silverstein and Boyer, *J. Biol. Chem. 239*, 3645, 1964; reproduced with permission of the American Society of Biological Chemists, Inc.)

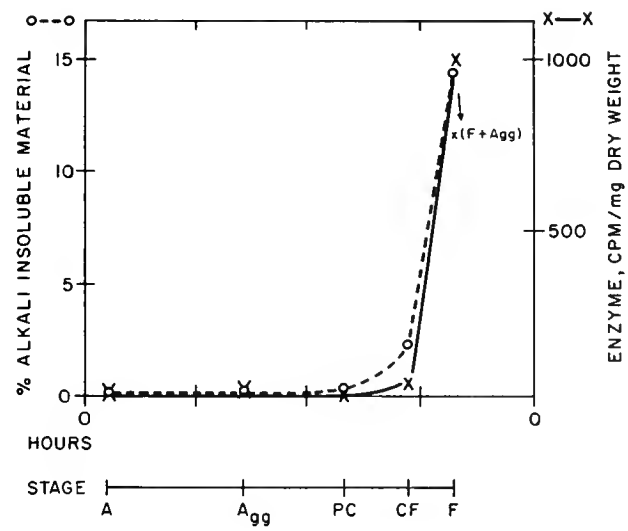

Fig. 8.

From Wright, Barbara, E.: Control of Carbohydrate Synthesis in the Slime Mold. In *Developmental and Metabolic Control Mechanisms and Neoplasia* (A Collection of Papers Presented at the Nineteenth Annual Symposium on Fundamental Cancer Research, 1965), p. 304. Baltimore, The Williams and Wilkins Company, 1965.

and is expressed on the right ordinate. Mixed preparations of an enzyme that was active with an inactive preparation gave relatively little inhibition. The data in Fig. 8 exhibit a striking correlation in the activity of an enzyme and the accumulation of the product of the activity of this enzyme. The correlation suggests a causal

relationship, but we shall see that this is not justified. We found that if one goes from $10^{-4}M$ EDTA to 0.1 *M* EDTA, it is possible to partially stabilize enzyme at the earlier stages of development and detect activity.

Table VIII is a stage study in which we harvested, killed the cells and isolated enzyme at two different stages, late aggregation and culmination, in the presence of 0.01 *M* EDTA and 0.1 *M* EDTA. In other words, we had four enzyme preparations: two at aggregation, two at culmination. The enzyme preparations were made and assayed as quickly as possible; then, they were stored in an ice bath at 5° and were assayed again at two hours and at 24 hours. As you can see, at both concentrations of EDTA the enzyme activity at aggregation decreased with time, but was spared to a greater extent at 0.1 *M* EDTA. At culmination, however, the enzyme activity was not only spared but increased. This enzyme is strikingly activated by high concentrations of EDTA. Clearly inactivation of the enzyme prepared at aggregation was more rapid between hour 0 and hour 2 than from hour 2 to hour 24. In other words, the inactivation curve drops rapidly at first and then more gradually. Seeing this, one wonders what happens between the time that the enzyme was first prepared and the time that the 0 hour value was obtained. In other words, in that period of preparing the enzyme inactivation may have been even more rapid. At any rate, enzyme prepared at one stage is more inactivatable than the enzyme prepared at a later stage.

We call this phenomenon differential inactivation. It is an *in vitro* artifact, fairly common in the slime mold. We are very impressed with the extreme difficulty of detecting it, since it took us almost a year in this case. In each stage study the period in time and in stage at which the enzyme activity can first be detected *and* shown to be unstable varies and is very short-lived. Until this enzyme is stabilized, we cannot determine specific enzyme activity as a function of developmental stage. Our experience with differential enzyme inactivation makes us very suspect of the *absence* of any enzyme activity and prone to place faith in changes in enzyme activity only when (1) the enzyme is detected and (2) is relatively stable or capable of being stabilized. It is possible that the cell wall enzyme under study is always present in the cell membrane but is undetectable *in vitro* due to the absence of stabilizing primer, for example. We have some preliminary data on this point, but before presenting it, I would like to summarize the facts briefly (Table IX).

We have recently found 4 enzyme activities involved in cell wall or glycogen synthesis or both (see Table IX). They may be the same enzyme or some of them may be different, at

TABLE VIII

Effect of EDTA Concentration on Initial Enzyme Activity and Stability[a]

Stage	EDTA molarity	C.P.M. 0 hr.	C.P.M. 2 hr.	C.P.M. 24 hr.
Late aggregation[1]	0.01	10	5	5
" "	0.10	117	59	35
Culmination[2]	0.01	28	75	112
" "	0.10	169	210	216

[1] 0.35 mg dry weight

[2] 0.50 mg dry weight

[a] From Wright, Barbara E.: Control of Carbohydrate Synthesis in the Slime Mold. In *Developmental and Metabolic Control Mechanisms and Neoplasia* (A Collection of Papers Presented at the Nineteenth Annual Symposium on Fundamental Cancer Research, 1965), p. 308. Baltimore, The Williams and Wilkins Company, 1965.

least with respect to their location within the cell. Now, the enzyme we've been talking about until now is the one found in the cell husk fraction of the sorocarp and the acceptor for the radioactive UDPG is in the cell wall; the enzyme is bound to the acceptor. The product is cell wall. That's the alkali-insoluble complex of cellulose and glycogen.

Also, we have been studying for some time an enzyme in the 100,000 x g pellet. This is the typical glycogen synthetase using UDPG and it depends upon glycogen as primer. Now, if this enzyme preparation is coaxed, it will use alkali-insoluble cell wall material as acceptor. Furthermore, cell wall primer is a competitive inhibitor of glycogen synthesis. Thus, the same enzyme catalyzes both reactions. In Table X this enzyme is described. Here we see it is possible to use an enzyme in the cytoplasm of the amoeba to synthesize alkali-insoluble cell wall polysaccharides. This enzyme is in the 100,000 x g pellet and, as you can see, it's completely dependent upon G-6-P and primer, the primer being alkali- and cellulase-treated cell wall material. Finally, we have detected an enzyme in the amoeba cell membrane. This enzyme will use glycogen as an acceptor but is unable to use alkali-insoluble primer. It responds to EDTA in a manner similar to the cell wall enzyme (Table XI). Perhaps prolonged incubation of this amoeba cell membrane fraction with partially soluble acceptors, such as cellodextrins, will reveal a capacity to synthesize an insoluble product. It's our hope to determine if these 4 enzymes (Table IX) are all different or, perhaps, all the same except for their localization in the cell and the primer to which they are bound.

In summary, we've seen that no single event could possibly trigger cell wall synthesis since a complex array of primer, substrates, activators and enzymes are not only limiting but must interact to bring about the accumulation of cell wall material. The relative contribution of these factors and of RNA and genetic control as well as the time at which each acts relative to the differentiation process are questions for the future. The probable interaction and interdependence of all of these mechanisms presents a challenging problem, to say the least.

PAPACONSTANTINOU: Are the glycogen enzymes the ones responsible for the linkage of glycogen and cellulose later?

B. WRIGHT: Right.

TABLE IX

Enzyme Source	Acceptor of UDPG-^{14}C
Sorocarp cell wall	Cell wall (bound)
Amoeba pellet	Cell wall (added)
Amoeba pellet	Glycogen (bound and added)
Amoeba cell membrane	Glycogen (added)

TABLE X

100,000 x g Pellet Enzyme Donating to Alkali-Treated Cell Wall Primer

Condition	cpm
0.2 mg primer	1,395
0.1 mg primer	589
No primer	8
No G-6-P	11

TABLE XI

Amoebae Membrane Preparation Catalyzing Incorporation of UDPG-^{14}C into Glycogen.

	Total cpm	
EDTA	Day 1	Day 2
Absent	14	0
Present	555	223

PAPACONSTANTINOU: So, if it's the same enzyme, you're going to have to postulate some mechanism for the change in function?

B. WRIGHT: By the same enzyme I mean we may only be hooking on glucose in alpha-1, 4 linkages to the cell wall material. We're looking at an artificial system; in the cell the ratio is about 1:1 of cellulose to glycogen in cell wall material, but *in vitro* we get 80% of it in the glycogen fraction. So that when I'm talking about this 100,000 x g pellet enzyme, it may just be adding to the glycogen moiety of the cell wall material. However, that is an alkali-insoluble material because it is intimately associated with the cellulose. Now, there is a big problem about the origin of the insoluble

primer. We are going to look for enzymes which could accumulate cellodextrins during development.

PAPACONSTANTINOU: Does this cell wall preparation include both spore and stalk?

B. WRIGHT: Yes, we've looked at both and, from staining reactions with iodine and various other things, we feel that the glycogen moiety is present in both equally. Thus, we think that the material that Gezelius and Ranby studied was, in fact, the material we are studying. This could explain their description of amorphous cellulose, if it really was 50% amorphous glycogen.

PAPACONSTANTINOU: How can you postulate the linkage of the glycogen and the cellulose? How do you picture it?

B. WRIGHT: Well, we tried to separate them physically with urea and high salt concentrations, etc., with very little success. Maybe you could get a very tight physical binding between the cellulose and the glycogen.

PAPACONSTANTINOU: What I'm wondering is, is it possible that there's an enzyme that is actually attaching alpha-1, 4 linkages to some part of the cellulose in a straight line of beta-1, 4's?

B. WRIGHT: Yes.

PAPACONSTANTINOU: You have a free hydroxyl in the 6 position of the hexoses in cellulose and you may be getting an alpha-1, 6 to start off the glycogen which will then be a series of alpha-1, 4 linkages.

B. WRIGHT: We have preliminary evidence for contaminating maltose in the cellulose fraction and cellobiose in the glycogen fraction.

PAPACONSTANTINOU: Oh, fine.

B. WRIGHT: However, this is all very tentative because you don't know how clean the preparations are. There is a soluble fraction and an insoluble fraction, but in each there could be small amounts of the other that were not actually attached. The amount of the radioactive cellobiose is so small that we don't like to make any definite statements until we get more of it. Maybe we can trick the *in vitro* system into making more of the cellulose fraction and really analyze it.

GROSS: How much galactose is in the cell wall?

B. WRIGHT: I don't know. I guess Maurice Sussman has data on that. Now, his material is soluble, of course; ours is an insoluble polysaccharide. We've looked for galactose in our preparations and found none. This cell wall material has been accounted for by weight, and it is pretty well characterized as a 50-50 mixture of cellulose and glycogen.

GROSS: Well, where is the product of that UDP-galactose transferred?

B. WRIGHT: That's on the surface, isn't it?

HANKS: I believe it's associated with the cell wall.

B. WRIGHT: Yes.

CANTINO: Do you know anything about the average chain length of the glycogen?

B. WRIGHT: We are now doing that enzymatically with a combination of phosphorylase and amylo-1, 6-glucosidase, determining glucose and glucose-1-phosphate. We don't know yet.

CANTINO: I wondered whether it might be changing at the spore stage as compared to the other stages.

B. WRIGHT: We want to look into that and compare the cell wall glycogen, after it's been separated from cellulose, to the pellet glycogen. Perhaps the cell could be insolubilizing the pellet glycogen, so to speak, as a primer. It'll be interesting if the amoeba membrane enzyme is similar to the cell wall enzyme. It reacts to EDTA the same, and it may be that we can't detect it in its potential role in cell wall synthesis because of the lack of alkali-insoluble material. The enzyme may be there earlier, but not bound to insoluble material.

TS'O: I'd like to raise some, perhaps, naive questions which have been bothering me. In differentiation, probably the most interesting event is the decision-making process. You have discussed the enzyme-inhibitor levels and rate-limiting processes. I wonder, how do these relate to the *real* decision-making process?

B. WRIGHT: I think it is wrong to think in terms of *an* important decision-making phenomenon; I think this never exists. This is a very complex interaction of many things, and it's misleading to look for *the one* cause.

GROSS: However, that might be precisely why decision-making is absolutely important. There may be two alternative steps, two stable states, each self-stabilizing as it matures, but one small thing may be the deciding factor.

B. WRIGHT: However, here we have shown there are numerous small things that are deciding factors, since they are all limiting.

GROSS: Yes, but *in vivo* presumably only one of them is active.

B. WRIGHT: No. This is a very complex steady state situation which is as stable as it is and as reproducible as it is precisely be-

cause there isn't *one* thing that's going to be important. If there's a little bit lacking of one thing, another will make up for it. That's why I used this model of the ATP-glucose-hexokinase system.

TS'O: That's another question I have. What you're saying is that you have a pretty good idea about how the glucose and the ATP together maintain a stabilizing effect for a steady state. You would think in terms of differentiation, however, unless the state is allowed to change its course, presumably the dynamics of the cell would not allow you to jump from one stable state to another.

B. WRIGHT: Cell wall construction is a big jump. There is no alkali-insoluble material, and suddenly you've got alkali-insoluble material. Let's just start with cellodextrins. You've got cellobiose in the amoeba. More complex cellodextrins are slowly building up so now you get 6 or 7 glucoses in a chain. It's getting almost insoluble. At the same time G-6-P and UDPG levels are rising. Glycogen is being broken down more rapidly because inorganic phosphate is accumulating, and you get a big build-up of precursors. Trehalose is starting to accumulate, also. Magnesium is becoming available by the breakdown of something else. All these things occur together at about the same time, buffering each other an interacting with each other. When the UDPG level is very low, G-6-P comes to the rescue. There's clear data for that. All these things occur together at about culmination and suddenly we've got the insoluble chains of beta-linked material; and now, the glycogen primer is at a state where it can be used for cell wall synthesis and the enzyme is being transferred, or perhaps is in the cell membrane already. This is pure speculation, but all these things together now give us what we consider to be quite a jump. It's really not a "jump" at all.

GROSS: Yes, you're going to see a dramatic change at some point from a system in which the product is soluble to a system in which it is insoluble.

B. WRIGHT: Right, and this can be a very gradual build-up of ten different things in order to create what we call a very abrupt change.

CHALKLEY: Wouldn't this, then, suggest that this is a modification of the differentiation process rather than a complete new change from one differentiated cell to another differentiated cell.

TS'O: Your point seems to be the following: in your system there is a mainstream flowing through slowly and it is the accumulation of the stream that gives the momentum for this "abrupt" change. However, I think in many other systems - not being a biologist I couldn't give you specific examples - probably one could have a diversion of the stream, i.e., it can go one way or the other. It is the diversion of the stream, a new choice and not just a continuation, which I would consider a differentiation.

B. WRIGHT: You have to be more specific or we can't discuss this.

ZIMMERMAN: How would the antigen system that we have just discussed relate to this?

B. WRIGHT: There *are* differences in enzyme levels. Alkaline phosphatase, as I said, increases seven-fold.

EPEL: I think what Dr. Ts'o would like to know is, is there some point when you start initiating this? Is there some earliest point at which you synthesize a real enzyme?

PAPACONSTANTINOU: Well, aren't you going from a glucose-6-phosphate independent enzyme to a glucose-6-phosphate dependent enzyme?

B. WRIGHT: The low UDPG level requires the G-6-P.

PAPACONSTANTINOU: Your culmination stage is very much analagous to the glycogen phosphorylase story in muscle in which one has the regulation starting with the cyclase. It looks like what you've got here is a situation where you may have to go one step further and look for some kind of cyclic -3',5' AMP which is hormonally regulated.

B. WRIGHT: Oh yes. An nucleotide levels change also during differentiation. Now, if we could bring in the phosphorylase story which is very much involved, the reactions we've discussed may very well depend on glycogen breakdown. We could make it even more complicated. However, I think if we want to discuss the point we shouldn't complicate it further by bringing in more reactions.

PAPACONSTANTINOU: However, the point is that you've got, also, reaction dependence here.

B. WRIGHT: Right. There's an intense competition and interaction among all the reactions which are going on.

PAPACONSTANTINOU: My only point is this (I'll try and make it as simple as possible): it appeared to me that you were going from a system in which the enzymes showed more of a substrate independence to a differentiated state in which the enzymes showed more of a substrate dependence.

B. WRIGHT: No, I showed that G-6-P stimulated at low UDPG not high UDPG. This doesn't make the enzyme different. All I said was that the combination of increasing G-6-P and UDPG would enter into their effect on cell wall synthesis; and if the UDPG level happened to be unusually low, the G-6-P would stimulate the cell wall synthesis more. This is not involved in the reaction although it's a modifier. It stimulates cell wall synthesis more when UDGP is low, no matter what stage the enzyme is taken from.

PAPACONSTANTINOU: No matter what stage you take this enzyme from you always get the same reaction?

B. WRIGHT: Right.

GRUN: Am I wrong in thinking that this organism at the time that it is aggregating is a syncytium?

B. WRIGHT: That's wrong. There are individual cells.

FERGUS: There's still one nucleus per cell.

TS'O: I think that the kind of differentiation process which I have in mind is different from what you have described. For instance, I could pose a decision-making process like that in the determination of sex. Once the decision is made, the organism will carry this decision to its grave. That decision is made in the early cell and you cannot change it.

B. WRIGHT: I don't think you can consider such complex examples if we're going to talk about it. This is why I introduced the talk by saying morphogenesis is a change in structure; therefore, we can look for a simple example that we can talk about. A lot of biologists like to talk and think in such complex terms about morphogenesis, it's difficult to analyze it.

GROSS: Are there any specific differences where you can find rate-limiting reactions or something that does *one* thing that does give control such as induction in *E. coli?*

B. WRIGHT: UDPG is limiting, so is G-6-P, and several other things here are also limiting. If you studied one of them alone, it might look as though you'd found the answer.

GROSS: The general trend of what you're saying is that epigenetic considerations may be more central to differentiation than genetic ones.

B. WRIGHT: No, if you don't have the gene, you don't have the enzyme. All I'm saying is that if you want to know what the *immediate* control of this process is, it may or may not be genetic: you may have gotten the synthesis of the relevant enzyme a long time ago; you may already have the enzyme, or at least the message for it. In glutamic acid dehydrogenase you have the enzyme throughout the differentiation, but it becomes 7 times more active when it gets more endogenous substrate. This is another thing I think ought to be stressed to clarify the situation. What we are talking about when we say: *this* is essential; *this* is important. This is one minor cause here, and we're just beginning to clearly see that you may have a lot of causes at one time. You know before a particular differentiation process, for example, you've got templates. They are one kind of cause. Now, where does the immediate control lie? Maybe it's on the activation of the message. Maybe that's not it at all. Maybe the enzyme is synthesized all the time and it simply accumulates because the substrate is stabilizing. There could be other explanations, and are probably many of them.

TILL: Am I right that you're arguing that what you've studied is all an inevitable consequence of the starvation?

B. WRIGHT: We know that definitely; if it gets fed, it doesn't differentiate.

TILL: Then the decision is whether or not it gets hungry.

CHALKLEY: It's the concept of differentiation that we're mixing up. Differentiation at the epigenetic level defined in terms of morphological changes. One of you is talking about that and one of you is talking about genetic control in an already differentiated system.

B. WRIGHT: They're both essential and we should just define which one we're talking about. I think it's important to stress that there is no one important thing here at all.

FERGUS: I don't think that fruiting necessitates starving because you can obtain fruits right on the same plate with a large supply of bacteria still present.

B. WRIGHT: Yes, but they're not eating it.

FERGUS: Well, if they aren't eating, there must be some other factors, then, that prevent their ingestion, rather than that they're being starved. They're not being starved; they're already full of bacteria.

B. WRIGHT: When they're aggreating, they are essentially starving. They'll do the same thing whether you have them on nutrient agar or 2% agar. An important factor in their starvation may be that the permeability is terrible in these amoebae. The permeability for some compounds is 1/20th as good in the amoeba as it is at culmination; by that time you can't interest them at all in eating.

FERGUS: I'm sorry; you left me. There are bacteria present, and these cells can ingest bacterial cells.

B. WRIGHT: Yes, there are bacteria that these cells like.

FERGUS: All right, they're still there because you can get sorocarps with plentiful numbers of bacterial cells present.

B. WRIGHT: No!

KAHN: No, I agree; you can't!

FERGUS: You certainly can; I've been able to do it.

B. WRIGHT: They're not the kind of bacteria that these cells like.

DEERING: It's a question of whether they're taking the bacteria up or not.

FERGUS: Well, then there is some factor that is controlling the failure of the amoebae to ingest.

DEERING: If you plate the myxamoebae out on a lawn of bacteria, you can get colonies of aggregation and culmination with bacteria between the colonies. If you put only a few amoebae down on a plate, they will divide and then go to a final stage, but there will still be bacteria that will be physically inaccessible to them. You get clear regions in the bacterial lawn that have been eaten out by the amoebae.

GREGG: You can get aggregation among bacteria; there's no question about that. Probably the differentiation mechanism overrides the feeding one; you get aggregation in the presence of bacteria, and they stop feeding at that time.

TS'O: Back to the original controversy. Usually, I would think one of the chief purposes of people working together in development is trying to find the most important factor which determines why a certain event will occur in a certain way. On the other hand, some may think that all factors involved are equally important. It seems to me, therefore, there is a fundamental difference in philosophy and that's what we are arguing about and what this workshop is about.

B. WRIGHT: It certainly is. It's a very fundamental difference because people go looking for *the* cause of morphogenesis when there are many.

TS'O: It's naive, but the systems we're working with clearly ask that question.

B. WRIGHT: We have not picked small enough problems to be able to find out wnether it's naive or not. I mean, if you look at some gross change, if you look at a sea urchin egg, you know nothing about what's going on during metabolism. Here in the slime mold, it's so simpleminded that the main thing it's doing is converting protein to carbohydrate, and you can study a simple reaction in this process and this has some meaning. If you attack a complex system, you will not know what questions to ask, or get around to knowing the answer to the questions, because you don't know enough about the thing you're studying.

GROSS: But suppose, for the sake of argument, that somebody were interested in hemoglobin synthesis. It's a very complicated system. Suppose you're lucky enough to show that at a certain time in the development of a chick, for example, product x is to come off the shell. This product becomes soluble and is a specific inducer for the messengers that are involved in the heme part of hemoglobin. Hemoglobin begins to be synthesized and that, in turn, is responsible for the aggregation or the differentiation of the blood islands.

B. WRIGHT: All right, you can make an isolated observation like that and in this complicated system that's as far as you'll go with it.

TS'O: The question in my mind is whether or not this organism has made an internal decision at this point to start differentiating or just that it starts to differentiate when it has used up its food. Look at all the synthesis of the cell wall material. A tremendous amount of chemical energy is being used there.

B. WRIGHT: There are many processes begun when it starts starving at 0 hours and at 15 hours it makes cell wall; if you look at what's going on inside there, you see the proteins decreasing, the amino acid pool is diminishing, the glucose is increasing, and the cell wall is being made.

KAHN: Pseudoplasmodia (slugs) can under the appropriate conditions migrate for several days. It is not until the slugs cease migrating that final cytodifferentiation begins. Clearly, the "cue" which triggers differentiation cannot be "starvation" alone.

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CELL INTERACTIONS IN SLIME MOLD (ACRASINA) DEVELOPMENT

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Ontogenetically meaningful exchanges occur between cells and tissues during the development of all multicellular organisms. In the discussion to follow, evidence will be presented that cellular slime molds are not exceptional in this regard.

The now rather familiar life cycle of cellular slime molds is shown in Fig. 1. While this drawing was prepared to illustrate *Dictyostelium purpureum*, in essential features, it is representative of all members of the family *Dictyosteliaceae*. Our attention today will be focused upon the early stages of the cycle, beginning with spores (Fig. 1A) and terminating with the formation of the pseudoplasmodium (aggregate) (Fig. 1D).

A nutrient agar plate inoculated with bacteria and *Dictyostelium* (or *Polysphondylium)* spores will generally show all stages of the life cycle after two or three days of incubation at 25°C. To obtain greater developmental uniformity, amoebae may be pregrown in liquid culture, harvested at the end (or during) the growth phase, washed and dispensed upon a non-nutrient substrate. *D. purpureum* amoebae, under these conditions, begin to aggregate after a few hours and complete development in less than twenty-four hours. Furthermore, since most of the cells aggregate at about the same time, substantial synchrony is achieved.

In many respects, a spore is like a zygote--each spore possessing the ability to germinate, grow and develop into a complete multicellular unit. Thus, if spores are isolated and cultivated, genetically pure clones can be derived.

Table I presents a summary of the various types of cellular interaction that have been detected during early slime mold development. Some of these phenomena are much better known than others but all, I believe, are worthy of inclusion in this survey.

The first item in Table I indicates that spores may interact to limit spore germination. Russell and Bonner (1) showed that a significantly higher percentage of germination occurs in sparse (dilute) groups of spores than in dense groups. There are two possible explanations for this observation. When a spore germinates, it may release into the environ-

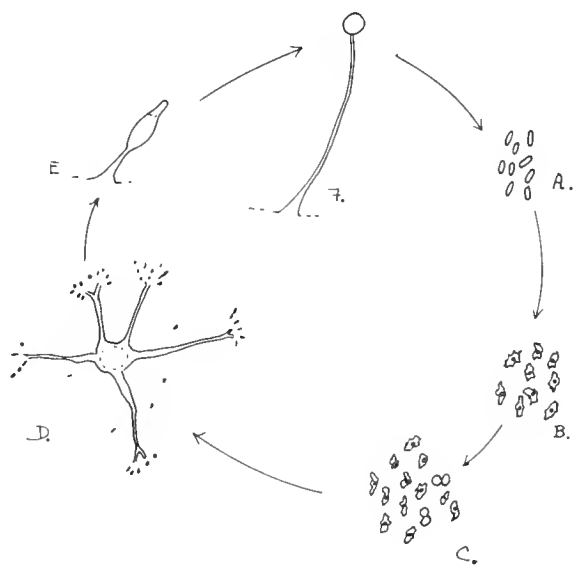

Fig. 1.

Life cycle of cellular slime molds. A) Spores; B) germinated amoebae; C) feeding and cell division; D) aggregation; E) culmination (differentiation of fruiting body); F) sorocarp (fruting body) consisting of stalk and spore mass (sorus).

TABLE I

The Types of Cellular Interaction Which Take Place During the Early Development of Slime Molds.

CELLULAR:

SPORE	⇌	SPORE (INHIBITION)
BACTERIUM	→	SPORE (STIMULATION)
BACTERIUM	→	VEGETATIVE AMOEBA (ATTRACTION)
VEGETATIVE AMOEBA	⇌	VEGETATIVE AMOEBA (REPULSION)
AGGREGATIVE AMOEBA	⇌	AGGREGATIVE AMOEBA (ATTRACTION)

Implemented by

A) Chemotaxis; relay amplification

B) Contact following; adhesion

MULTICELLULAR:

CENTER	⇌	CENTER (INHIBITION)

ment a spore germination inhibitor. Or, spores may compete for some essential factor during germination. Thus, the first spores to become active would remove this factor from the environment and limit the germination of the remaining spores. No evidence is available to distinguish between these two possibilities.

The next item in Table I suggests that bacteria may stimulate spore germination. The evidence for this phenomenon is limited to some observations that I made several years ago. I found that six to ten times more spores would germinate in the presence of bacteria than in their absence. How bacteria influence germination is, unfortunately, not known.

As is indicated by the next item in the table, bacteria may also influence the movement of amoebae. Samuel (2) demonstrated that amoebae migrate toward bacteria probably in response to a chemical released by the bacteria. The possible relationship of bacterial-amoebal chemotaxis to aggregation is of interest. It is well established that aggregation in cellular slime molds is largely the result of chemotaxis. Therefore, during the evolution of these organisms, chemoreceptors must have evolved for the receipt and translation of chemical signals. The first receptors were probably used to detect and capture bacteria. If this is so, then perhaps the receptor(s) that operates in aggregation might be nothing more than a modified version of that used to detect bacteria and, as such, still is somewhat sensitive to bacterial attractant. This last assumption could account for the observed absence of aggregation in the presence of bacteria. Since the attractant released by the bacteria would compete for or occupy receptor sites, no clear aggregation signal could be received until the bacteria were removed.

The next item in Table I indicates that vegetative amoebae repulse one another. Samuel (2) found that if amoebae are dispensed in small, dense groups on an agar surface, they will migrate from the group along rather direct paths. This migratory activity is probably the result of a "repellent" that accumulates when vegetative amoebae are present at high density.

Aggregation is the most complex series of interactions that takes place in early slime mold development. It is characterized by the formation of migrating streams of cells (Fig. 1D). Stream formation is the result of two mechanisms; chemotaxis (and related "relay amplification") and "contact following." Relay amplification describes Shaffer's model of slime

mold aggregation. In this model, the attractant (acrasin) produced by one cell causes adjacent cells to migrate toward the source of acrasin and to, in turn, produce acrasin. The acrasin produced by the affected cells stimulates other cells to do likewise resulting in the "relay" and "amplification" of the aggregation message. Contact following is a term used by Shaffer (3) to indicate that cells in a stream adhere and follow one another. Like circus elephants, the cells in a row follow the lead cell. How information regarding speed and direction of movement is relayed from cell to cell is not known.

Our attention, to this point, has been focused on those interactions which take place between cells. The final item in Table I refers to an interaction at the multicellular level. This interaction is manifest in the disposition of centers (centers of aggregation) with respect to one another. More precisely, certain evidence indicates that the presence of one center may dictate whether a second center can form within the immediate area.

Before discussing this phenomenon, are there any questions?

GREGG: Arnold, would you care to comment on the fact that you can get aggregations within a mass of bacteria on occasion?

KAHN: I haven't seen this occur myself, but I can think of a possible explanation. If the bacterial attractant is short-lived (acrasin is short-lived under normal conditions), then a point may be reached where it would no longer compete with acrasin and aggregation could proceed.

GRUN: If you take an amoeba from a colony which is aggregating and if you put it into the middle of a colony which is vegetative, does it pass the message to the others?

KAHN: No. However, Sussman has shown the aggregative phase amoebae can stimulate aggregation in developmentally younger cells.

If there are no further questions, I should like now to return to the last item in the table. My interest in this problem arose as the result of several investigations carried out by Bonner and co-workers (4-6). Their studies indicate that the orientation of fruting bodies and the number of aggregates formed per unit area of substrate may be under the control of a factor present in the gaseous phase of the environment. They termed this factor the "spacing substance."

I began my study in the hope of answering two questions. First, does the spacing of aggregates occur in *Polysphondylium pallidum?* Second, if such spacing does occur, is it the result of a spacing substance present in the gaseous phase of the environment? Previous work with *Polysphondylium* indicated that this species was responsive to those factors (charcoal, mineral oil) used by Bonner to reduce or eliminate the spacing substance.

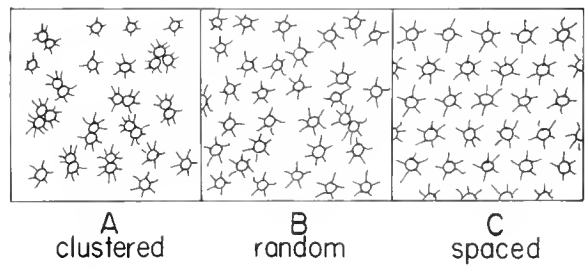

Fig. 2.

Three types of possible spatial distribution of aggregation centers. A) Clustered, centers appearing in groups; B) random, centers distributed as expected on the basis of chance; C) spaced, centers placed at equal distances from one another. The density of centers is the same in all three examples.

Spacing may be defined as the distribution of centers of aggregation on a substrate. A "spaced" distribution is one in which the centers tend to form at equal distances from one another (Fig. 2C).[1] A "clustered" distribution, on the other hand, is one in which the centers tend to appear in groups (Fig. 2A). The method of Clark and Evans was used to determine the distribution of centers. This method consists of calculating the nearest neighbor distance expected if the distribution is at random and comparing this value with one derived by actual measurement. If the distribution of centers is random, the ratio of observed to expected is unity. If the distribution is spaced, values greater than one are derived; if clustered, the values are less than one. Figure 3 is a graphic illustration of the relation between nearest neighbor distance and the density of aggregation centers. Note that deviations to the right of the curve indicate a spaced distribution while deviations to the left indicate clustering.

In these experiments, the cells were pregrown in liquid culture, washed free of residual bacteria by differential centrifugation, suspended in a saline solution, and dispensed in

[1] Figures 2-6 are sketches of data which will appear in Developmental Biology, 1966.

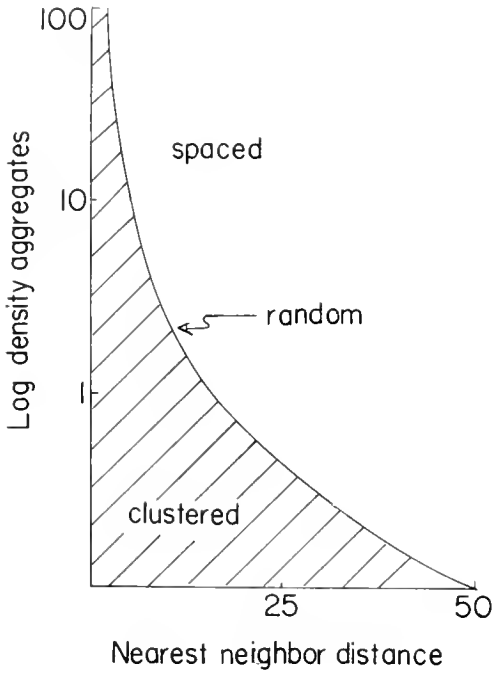

Fig. 3.

The relation between the density of aggregates and nearest neighbor distance. The curve depicts the relationship expected if the spatial distribution of centers is at random. Deviations to the right of the curve indicate a "spaced" distribution; deviations to the left, a "clustered" distribution.

drops on buffered non-nutrient agar. Counts of the number of aggregates were made in all cases after 24-26 hours of incubation. In some cases, counts were also made at hourly intervals to determine the rate of center formation. Nearest neighbor distances were obtained with an ocular micrometer.

When counts and measurements were made on a number of aggregating populations, it was found that all three types of distribution occurred. Random distributions were the most frequent, followed by spaced and then clustered. Since spaced distributions occur in the presence of charcoal (an agent that should remove the spacing substance), it suggests, but does not prove, that such spaced distributions *are not* the result of a gaseous spacing substance. Interestingly, spaced distributions were most often observed in low center density situations while clustered distribution were associated with high density.

The correlation between center density and distribution led to a consideration of those

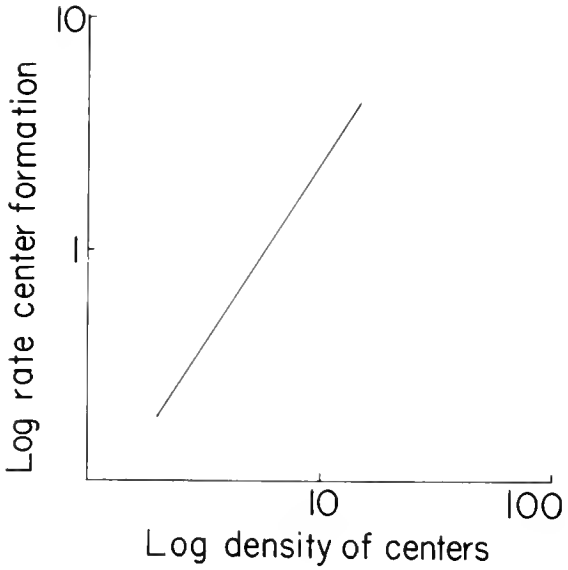

Fig. 4.

The relationship between the rate of center formation (the number of centers appearing per unit of time) and the final density of aggregation centers on the substrate (surface). Note that the faster the rate of center formation, the higher the final density.

factors or phenomena that determine center density. One, apparently fundamental, relationship is illustrated in Fig. 4. Note that the faster the rate of center formation, the higher the density of centers.

The next step, then, was to ascertain those factors which play a role in determining the rate of center formation. The influence of a number of such factors are shown in the graphs in Fig. 5.

Figure 5A illustrates the rate of center formation as a function of stage in the growth cycle. Note that stationary phase cells begin to aggregate the moment they are placed on the substrate, while logarithmic phase cells do so only after a lag of two hours. Furthermore, once aggregation begins, log phase cells proceed at a much slower rate than do stationary phase cells.

Amoebae which are incubated in the light and in the presence of charcoal or mineral oil, aggregate much faster than comparable amoebae incubated in the dark and in the absence of these two factors (Fig. 5B). Charcoal and mineral oil are believed to remove a center suppressing factor present in the environment while light is believed to mitigate the effect of this factor (7).

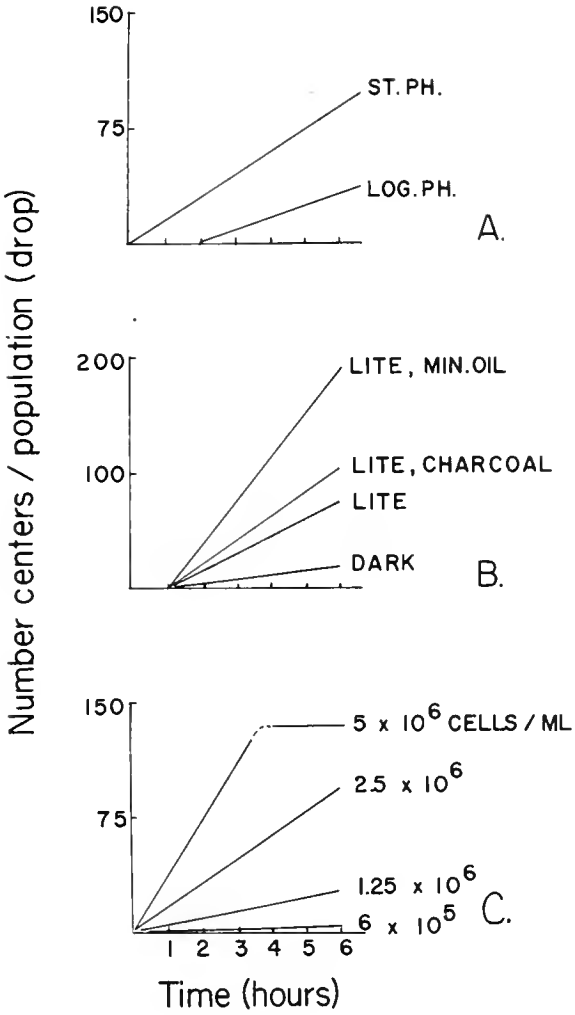

Fig. 5.

The influence of various environmental and biological factors on the rate of center formation. The data are plotted as the number of centers per drop (group or colony of cells) against time. Graph A indicates that cells taken from the stationary phase of growth aggregate sooner and at a faster rate than do logarithmic phase cells. Graph B illustrates that the rate of center formation is faster in the light and in the presence of charcoal and mineral oil than in the dark and in the absence of these two agents. Graph C shows that the rate of center formation is faster at high cell density than at low cell density.

Figure 5C shows the relationship between the rate of center formation and cell density. The higher the density the faster the rate. This result would be expected if increasing the density of cells also increased the number of cells ontogenetically ready to aggregate.

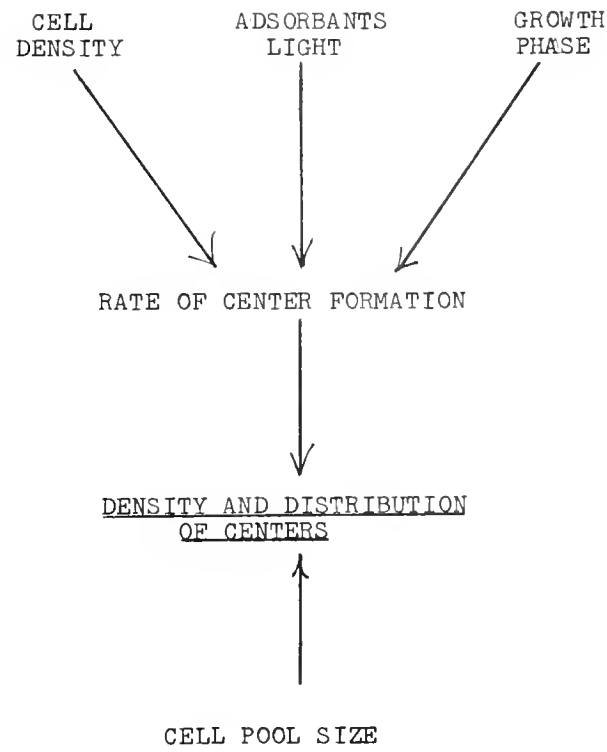

Fig. 6.

The inter-relationships between various environmental and biological factors, the rate of center formation and the distribution and density of centers. Note that the rate of center formation and cell pool size are the primary factors in determining center density and distribution.

Figure 6 summarizes the inter-relationships between the various factors that influence the rate of center formation, and the distribution and density of centers. One final variable, not previously mentioned, is "cell pool size", the number of cells available for aggregation. If the pool of cells is large, then after the initial wave of aggregation, the cells remaining could aggregate to form additional centers. This would result in an increase in center density and would favor the establishment of random or clustered distributions since these "secondary" centers could form at any distance from the first. Conversely, if the pool is small, few if any cells would remain after the first wave of aggregation and no secondary centers could form. This situation would minimize center density and favor a spaced distribution.

Two models satisfactorily account for the relationship between the rate of center formation, and center density and distribution. In one

model, it is proposed that an inhibitor produced by a center inhibits the formation of other centers in the immediate area. In the other model, no inhibitor is postulated and the distribution of centers is accounted for by the removal of cells, since without cells no centers can form.

In either model, the distribution and density of centers depends upon the area that initially formed centers control (either by withdrawing cells from the surrounding substrate or through the spread of inhibitor). Thus, if the time interval between the appearance of centers is long (a slow rate of center formation), a substantial area would be occupied and later appearing centers would be displaced at some distance from those centers that form first. This situation would favor a spaced distribution of centers and low center density (Fig. 7A). On the other hand, if the time interval is short, initially formed centers would have little opportunity to establish territories before other centers would appear. Since later appearing centers could form at almost any distance from the first, this situation would favor the establishment of random, if not clustered, distributions (Fig. 7B). While we cannot, with the data at hand, distinguish between these models, the cell withdrawal hypothesis is favored since it does not require the postulation of an additional, unknown factor.

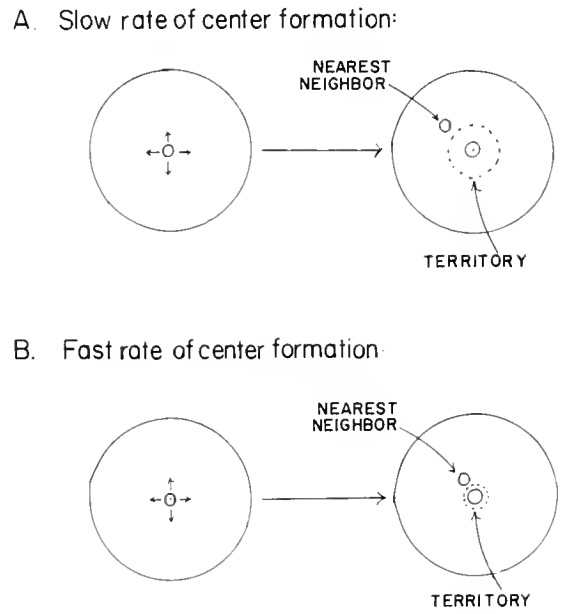

Fig. 7.

The consequences of the rate of center formation on center density and distribution. The faster the rate, the smaller the area (territory) controlled by first formed centers.

We may conclude, then, that the non-random distribution of centers (spaced or clustered) occurs in *Polysphondylium pallidum;* that center distribution is probably not the result of a "spacing substance" present in the gaseous phase of the environment; that what is involved in establishing center density and distribution is the rate of center formation and the number of cells available for aggregation.[1]

GRUN: It might be possible to find out whether there is an inhibitory substance or suppressor simply by taking strips of agar these are growing in from between the centers and putting them on a petri dish between strips of agar which have not had centers growing near them, "undifferentiated" agar, and then see if amoebae placed on this surface will stay off the experimental strips.

KAHN: Shaffer has done an experiment similar to the one you suggest. Aggregates were allowed to form on opposite sides of a thin agar membrane. Under these conditions, it was possible to note that aggregates tend to organize in the space between aggregates located on the opposite side of the membrane. This suggests that some sort of diffusable inhibitor (spacing substance) may be produced that determines the spatial distribution of aggregates.

GRUN: It would be diffusing upward in this case?

KAHN: Yes.

GREGG: Did you say that the centers form in between the original centers?

KAHN: Yes.

GREGG: How does this correspond to Sussman's thin membrane experiment?

KAHN: I don't know. The observations are certainly contradictory.

EPEL: Do these centers all have varying numbers of cells in them or does that vary under these conditions, too?

KAHN: In a rapidly aggregating population of cells, one tends to get numerous aggregates of "moderate" and approximately equal size. In a slowly aggregating population, fewer, but larger, aggregates are formed.

GRUN: You didn't talk about the mineral oil.

KAHN: No one really knows how mineral oil influences aggregation. Perhaps it is behaving as an absorbant (adsorbant?). Personally, I feel

[1] The data presented above will appear in full in Developmental Biology, 1966.

more confident about the effect of charcoal.

TS'O: I'd like to ask a question about the data on aggregation. Is there a possibility that some of the influencing substances are physical in nature?

KAHN: There's a very good possibility.

POLLARD: Has anyone tried to prevent this phenomenon in an electric field?

KAHN: No, but I think it would be a very good idea to check for possible bioelectric phenomena in aggregation. In a single trial, we *were* able to detect a potential difference between the front and back end of the slug.

POLLARD: However, if this thing is alternating very rapidly, perhaps you might not be able to interfere with it.

KAHN: The apparent rapidity of cell movement in aggregation (note: as seen in a film shown during this talk) is an illusion created by showing time lapse photographs at normal projection speeds. Actually cell movement is quite slow.

UNKNOWN DISCUSSANT: One last question while we're on this subject of potential. Has anyone tried the effect of chelating agents on this phenomenon?

KAHN: DeHaan did this with EDTA.

UNKNOWN DISCUSSANT: Wouldn't this interfere with the adhesion?

KAHN: It does. Apparently the aggregates formed without streams. That's why I think this ought to be looked at in detail.

GREGG: Gerisch also did this and he found an EDTA sensitive stage and an EDTA insensitive stage. After aggregation occurs, they're EDTA insensitive so they stick together.

UNKNOWN DISCUSSANT: Is there any morphological polarity in these cells?

KAHN: During aggregation, there is at least transient morphological polarity.

GREGG: Does your curve imply that founder cells may occur as a result of aging of the cell?

KAHN: "Developmental" age is probably one of the factors that plays a role in the establishment of a founder cell. In this case, the transition period between the end of feeding and the onset of aggregation is probably the most significant.

EPEL: Is there any possibility they're going anaerobic under mineral oil?

KAHN: Mineral oil does permit the diffusion of gases and you must bear in mind that the layer used in these experiments was not very thick.

GREGG: Well, won't they aggregate anaerobically anyway?

B. WRIGHT: Yes, but what is called anaerobic sometimes is not strictly anaerobic.

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HISTONES IN RELATION TO CONTROL IN LIVING SYSTEMS

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As this is a workshop, what I plan to do is provide a broad outline of some of the things which are being studied in Professor James Bonner's laboratory at the California Institute of Technology. We are concerned with the molecular aspects of control mechanisms in differentiated tissues. The strategy of attack is first to isolate the chromosomal material in a pure form.

In Fig. 1 is shown a general scheme for the isolation of chromatin. This scheme is applicable to mammalian tissues and slight modifications are necessary for plant tissue, but the general principle is the same. The tissue is disrupted in a Waring blendor in increasing volumes of the grinding medium and at increasing speeds. The grinding medium consists of: 0.25 *M* sucrose, 0.003 *M* calcium chloride and 0.005 *M* tris, pH 7.3. Grinding at increasing volumes and increasing speeds removes perinuclear contamination and gives rise to what we think are reasonably pure nuclei. These nuclei can be used for amino acid incorporation studies *in vitro*. The nuclei are washed once with grinding medium and then with saline EDTA. This inhibits the action of degrading enzymes and also removes the calcium that is stabilizing the nuclear membranes. This makes the next step, lysis in 0.01 *M* tris, more convenient. The lysed material is centrifuged through a rough sucrose gradient at 22,000 rpm for two hours. This gives rise to a gel-like pellet which, after dialysis against low concentrations of tris at pH 7.3, is known as "purified chromatin". Chromatin so prepared has a high Svedberg constant and for the purpose of a number of experiments it has proved advantageous to shear the material and remove larger aggregates by low speed centrifugation. The nucleoprotein remaining in solution (90%) is commonly referred to as nucleohistone.

The chemical compositions of some of the chromatins that have been isolated are shown in Table I. The histone:DNA ratio is roughly 1:1. In addition there is a very small amount of RNA which is difficult to remove. This RNA is partially resistant to RNase (1, 2). In the case of pea cotyledon there is a more than normal quota of RNA, but one has to recognize that it is a rapidly developing system. It has also been impossible to remove all of the non-histone protein and this may have an important contribution to make toward the chromosomal apparatus. The histones themselves are acid-soluble and this frequently provides a method for their isolation. The molecular weight of the acid-extracted material appears to be less than 10^5. The molecular weight of lysine-rich histones is usually estimated to be about 10,000

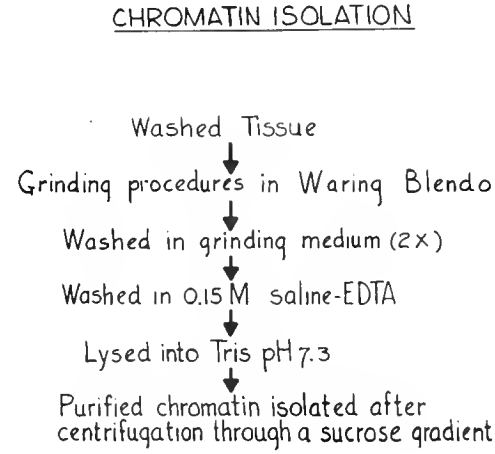


Fig. 1.

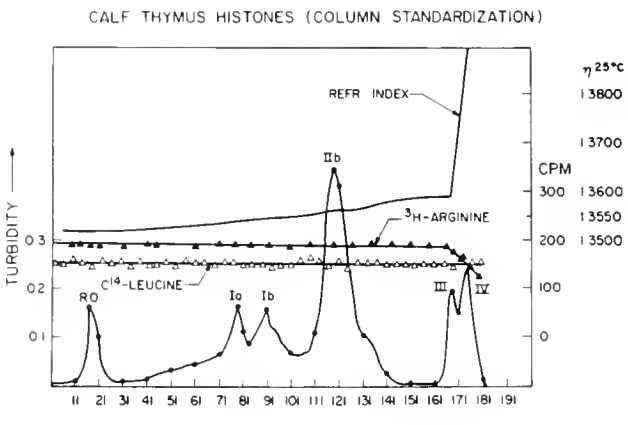


Fig. 2.

Elution of histones from the cation exchange resin GC-50, using a gradient of guanidinium chloride (8-40%, measured as refractive index). The quenching of C^{14} (△) or H^3 (▲) during the increasing salt concentration in the eluted fractions is shown.

and the arginine-rich histones are somewhere in the order of 25,000 (3). Elevated ionic strengths dissociate histones from the chromosomal apparatus (4). They contain neither tryptophan nor cysteine (5). Within a given species the electrophoretic pattern obtained on an acrylamide gel is comparable from organ to organ, but between species there are sometimes small but characteristic changes in patterns. Histones can be identified further by elution from a cation exchange resin, and this has proven to be a very useful tool.

A typical pattern is shown in Fig. 2. This shows acid-extracted histones from calf thymus. They were applied to the resin in 8% guanidinium chloride and eluted with the gradient shown. One invariably finds a run-off peak (R.O.), the nature of which is a matter for considerable speculation right now. Histones Ia and Ib are very lysine-rich, while IIb is moderately lysine-rich and III and IV are arginine-rich.

One of the earlier studies done in the group was to see if the isolated chromosomal material could do some of the things that one would expect of the *in vivo* material. One of these was to see if it could act as a template for DNA-dependent RNA synthesis and to compare the template activity of the chromatin with that of DNA which had not been isolated from an identical preparation of chromatin (Fig. 3). Here one sees the

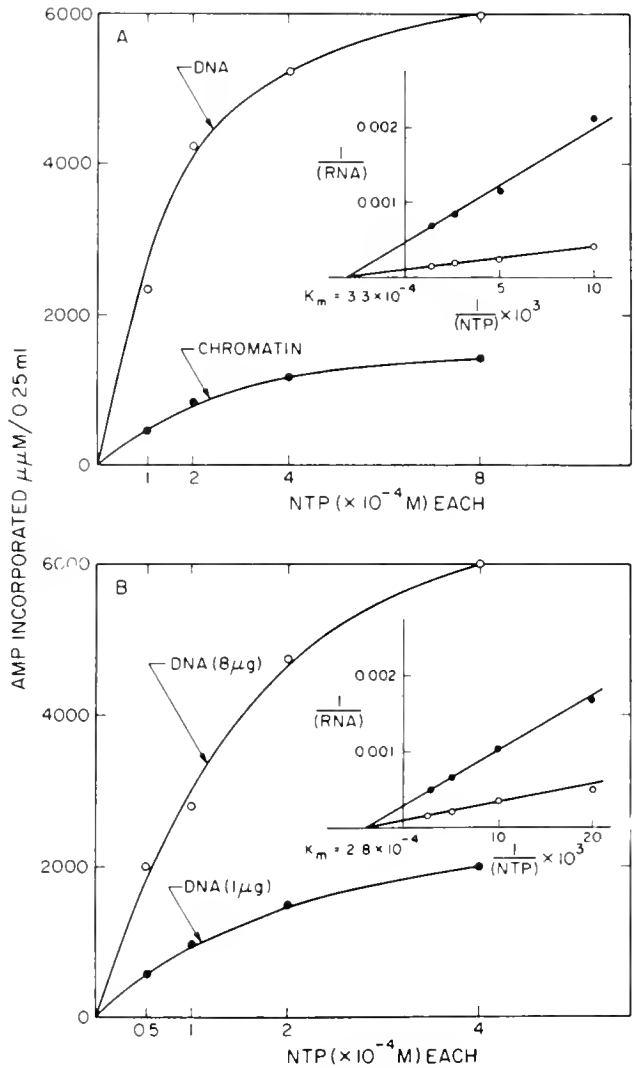


Fig. 3.

DNA-dependent RNA synthesis - a comparison of the template activities of liver chromatin and rat liver DNA. (a) The effect of increasing nucleoside triphosphate (NTP) concentration upon the template activity of DNA and chromatin (present in equal amounts); (b) the effect of increasing NTP concentration upon the template activity of different concentrations of DNA. (Fig. 7, Marushiga and Bonner, *J. Mol. Biol. 15*, 160, 1966; reproduced with permission of Academic Press.)

incorporation of AMP into RNA using as template either chromatin or DNA isolated from an equivalent batch of chromatin. It appears that the chromatin is unable to make RNA at the same rate as an equal amount of DNA. These particular experiments were performed by Dr. Marushiga. In Table II you see some more similarities with *in vivo* experiments. The synthesis

TABLE I

Chemical Composition of Chromatin (mass ratios)

	Calf Thymus	Calf Endometrium	Rat Liver	Pea Embryo	Ascites Tumor
DNA	1.0	1.0	1.0	1.0	1.0
RNA	0.022	0.11	0.043	0.55	0.12
Histone	1.14	0.91	1.0	1.07	1.20
Non-histone protein	0.33	0.66	0.67	0.57	0.98

of RNA is seen to be sensitive to actinomycin D. It is reduced in the presence of DNase but as the chromatin is fairly resistant to DNase the effect is more striking following pre-incubation of the chromatin with DNase.

Now, we know there are histones associated with this template, and it was intriguing to see if an appreciable quantity of histone could be removed without dissociating too much of the non-histone proteins that were present. So Dr. Marushiga examined salt extraction of rat liver chromatin and I show the results of some of these experiments in Fig. 4. He extracted with sodium perchlorate. First of all, as the concentration of sodium perchlorate increases, histones are released. Then in the region at about 0.4 *M* he began to dissociate a sizeable amount of non-histone protein. If one looks at salt concentrations where there is not a great deal of non-histone protein removed, but a considerable amount of histone is removed, it is possible to see in all three of these cases that RNA synthesis in the *in vitro* experiments has been considerably elevated. There appears to be a positive correlation between histone removed and increase in RNA synthesis.

Does the RNA made in these *in vitro* systems have any biological significance? Can it direct protein synthesis? In Table III you see the results of some work done by Dr. Bonner and Dr. Huang. They isolated chromatin from two sources: the pea cotyledon and from pea apical buds. They used the chromatin to generate RNA and then coupled it with a full protein-synthesizing system. Then, after incubation, they used immunological precipitation techniques to detect the formation of globulins. In the case of cotyledons which make globulin *in vivo*, the chromatin is able to direct (via RNA synthesis)

TABLE II

Inhibition of the RNA Synthesis Directed by Rat Liver Chromatin

No. experiment	System	AMP incorporated μμM/0.25 ml incubation mixture
I	Complete	710
	" + Actinomycin D (5 μg)	128
	" + DNAase (5 μg)	416
	" - chromatin	30
II*	Complete	1640
	" + DNAase (5 μg)	225
	" - chromatin	60
III	Complete	860
	" + RNAase (2.5 μg)	288
	" - chromatin	105

*Chromatin was preincubated with DNAase at 37° C for 10 min.

(Table 3, Marushiga and Bonner, *J. Mol. Biol. 15*, 160, 1966; reproduced with permission of Academic Press.)

a reasonable percentage of globulin synthesis. However, the apical bud chromatin, when treated in identical circumstances, appears to be able to synthesize only a very small amount. They ran some parallel experiments with T4 phage (which so far as we know do not make pea seed globulin) and the cross reaction they obtained would suggest a low background effect comparable to that obtained from apical bud chromatin. Apical buds *in vivo* do not make pea seed globulin. Thus there is a correlation between the protein made by a specific tissue and the ability of chromatin derived from that tissue to make messenger for that protein.

PAPACONSTANTINOU: Why is there that variability in the amount of protein that is being synthesized in the first column? You have about

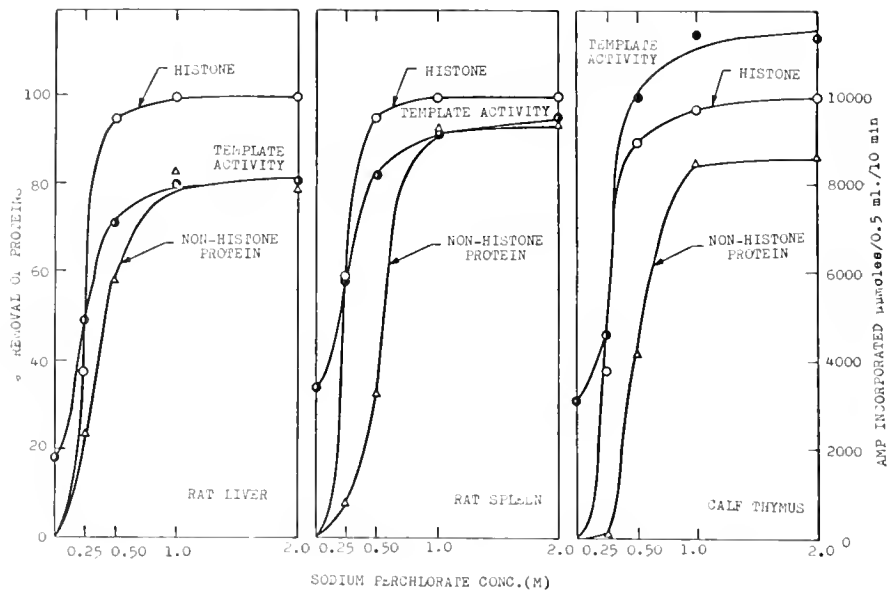


Fig. 4.

Relation between protein dissociated from rat liver chromatin and template activity (AMP incorporated). (Fig. 4, Marushiga and Bonner, *J. Mol. Biol. 15*, 160, 1966; reproduced with permission of Academic Press.)

TABLE III

Synthesis of Pea-Seed Globulin by Messenger RNA Dependent Ribosomal System in Response to Messenger RNA Generated by Two Different Kinds of Pea-Plant Chromatin

Template for RNA synthesis†	C^{14}-leucine incorporated into protein: Total soluble protein (cpm)	Globulin (cpm)	Globulin/total protein (%)
Apical bud chromatin	15650	16	0·10
Apical bud chromatin	41200	54	0·13
Cotyledon chromatin	8650	623	7·2
Cotyledon chromatin	6500	462	6·9

† The reaction mixture contains all materials required for both RNA and protein synthesis. Incubation for 30 minutes at 37°. All particulate material was then centrifuged off at 105000 · g and pea-seed globulin content of soluble protein synthesized determined by immunochemical assay.

five-fold difference when comparing the cotyledon chromatin and the apical bud chromatin. Do the preparations vary that much?

CHALKLEY: Well, I suspect that in these cotyledons the *overall in vivo* synthesis of RNA may be considerably below the overall RNA synthesis in apical buds and this may be mirrored in the capacity of the chromatin to make RNA. The results also reflect different scales of experiments.

PAPACONSTANTINOU: Did you treat with perchlorate?

CHALKLEY: No, that's exactly as it was isolated. They haven't been treated at all.

In order to identify the protein as precisely as possible they applied the following approaches. The *in vitro* synthesized protein was purified using the procedure applicable to pea seed globulin. If this material is synthesized from C^{14}-labeled amino acids and then diluted with cold globulin isolated from pea cotyledon, it has been shown to have exactly the same Svedberg constant as native globulin when studied in the ultracentrifuge. In addition, radioactive globulin was digested with trypsin and the resulting peptide fragments separated by two-directional paper chromatography, and compared with the tryptic digestion pattern from native pea seed globulin. Radioactivity was found in positions corresponding to every peptide spot (from native globulin) and no trace of radioactivity was located in other regions of the chromatogram. So it appears that it is really genuine globulin.

POLLARD: Have you done anything with antibodies? Does it precipitate?

CHALKLEY: Yes.

Dr. Maurer and myself have been concerned with the problems of histone metabolism. This is an interesting field in which the results tend to depend upon the methods employed for the isolation of the different histone fractions. We have been fortunate to have the advantage of using the relatively standardized IRC-50 microseparation techniques which have been developed at Cal Tech. In order to keep the system as simple as possible, we decided to investigate systems in which no DNA was being made. In the presence of DNA replication, as the DNA: histone ratio appears to be efficiently maintained, all histones must be synthesized. One promising system that we had available to us, in connection with some hormone studies which we were doing, was the endometrium tissue isolated from immature calves. We can get a great deal of this tissue which, in the absence of estradiol treatment, is not involved in DNA replication. The tissue was incubated in the presence of C^{14}-leucine and histones were isolated directly from the nucleus by acid extraction. The results of the subsequent fractionation of the histones are shown in Fig. 5. Again the optical density pattern is similar to that described previously. However, the most striking thing is that histones Ia, Ib, and IIb, so far as we can see, do not incorporate radioactive label to any significant degree. Peaks III and IV and the material in the run-off peak (R) do incorporate label. In order to demonstrate that this was standard protein synthesis, we ran control experiments with puromycin present in the incubation medium and the incorporation was decreased by a large amount in both instances (Fig. 6).

We were interested in seeing if this labeled histone was really attached to the chromatin which we isolated following procedures described earlier. We combined this with the study of the incorporation of amino acids into peptides in isolated nuclei. Figure 7 shows the results of a nuclei incubation followed by chromatin isolation from the nuclei. Histones were obtained by acid extraction of the chromatin. Again, there is incorporation of the label into III and IV and into the run-off peak. I should add that if one measures the specific activity in the peaks from the whole tissue incorporation, the specific activity approaches that of the whole cytoplasmic protein.

We wondered if this was a general effect or whether it was just a rather unusual result found in the one tissue. In Fig. 8 you see what

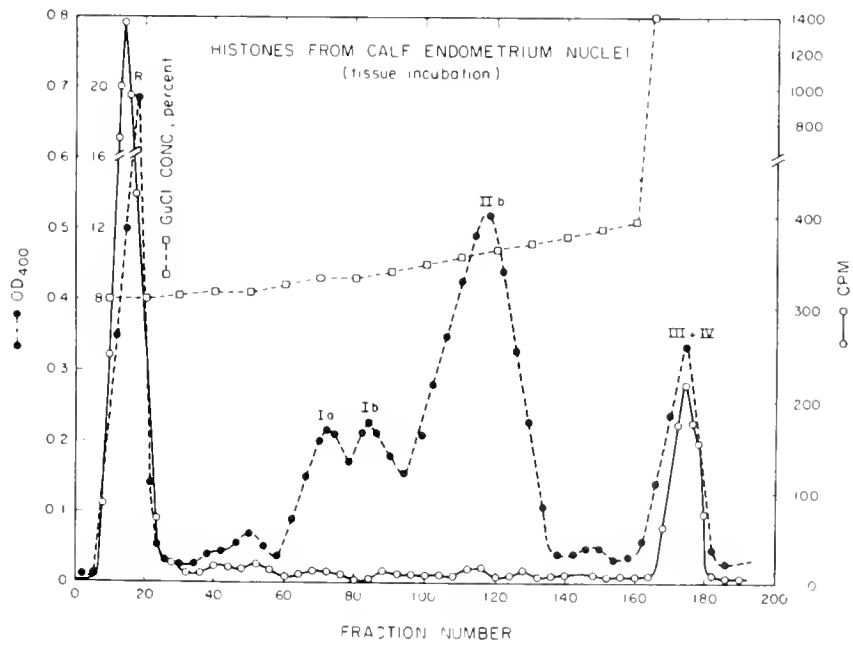


Fig. 5.

The biosynthesis of histones of incubated endometrium tissue in the absence of DNA replication. (Fig. 2, Chalkley and Maurer, *Proc. Natl. Acad. Sci. U.S. 54*, 498, 1965; reproduced with permission of the National Academy of Sciences.)

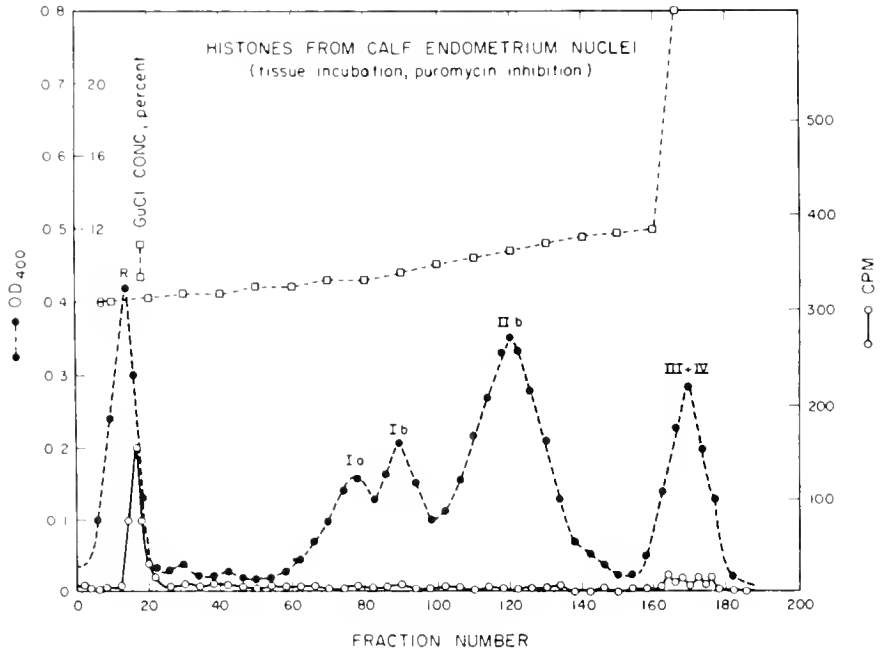


Fig. 6.

Puromycin inhibition of histone biosynthesis in the absence of DNA replication. (Fig. 3, Chalkley and Maurer, *Proc. Natl. Acad. Sci. U.S. 54*, 498, 1965; reproduced with permission of the National Academy of Sciences.)

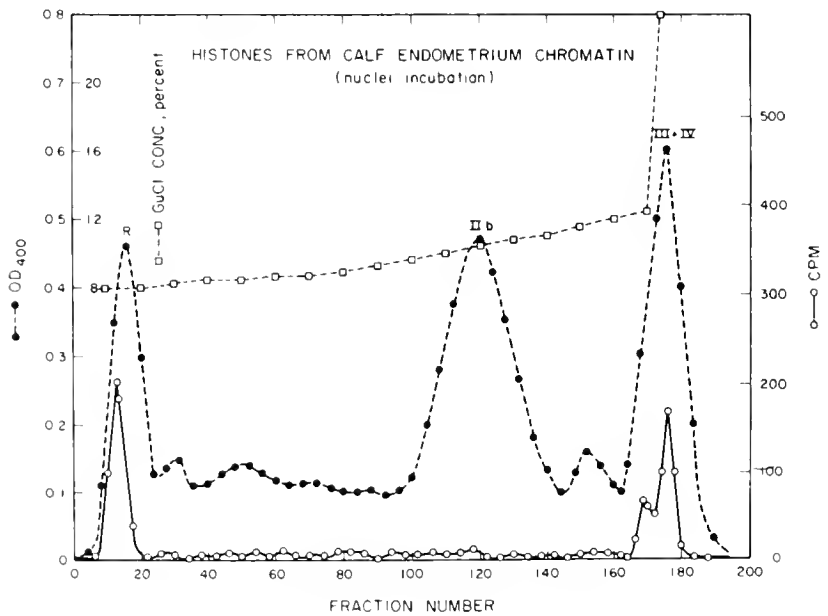


Fig. 7.

Biosynthesis of histones in incubated, isolated nuclei. (Fig. 1, Chalkley and Maurer, *Proc. Natl. Acad. Sci. U.S. 54*, 498, 1965; reproduced with permission of the National Academy of Sciences.)

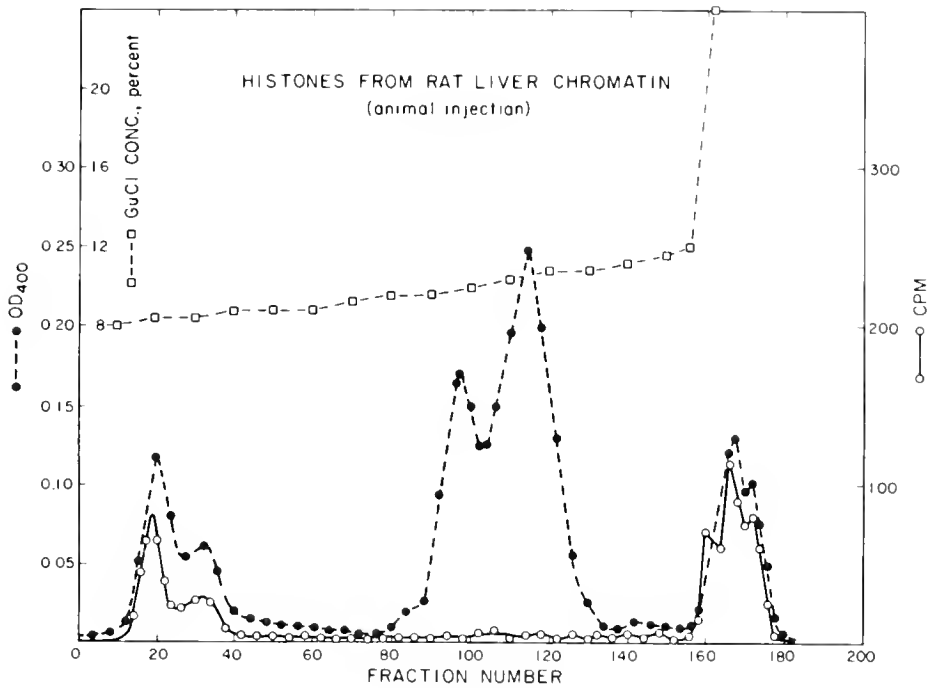


Fig. 8.

Biosynthesis of rat liver histones. (Fig. 5, Chalkley and Maurer, *Proc. Natl. Acad. Sci. U.S. 54*, 498, 1965; reproduced with permission of the National Academy of Sciences.)

happens if we inject C^{14}-leucine into a rat and isolate histone from liver chromatin. The first thing I would like to point out here is that the column elution pattern of histones has now changed a little. This isn't surprising as slight variations are found from species to species; though the similarities between histones are often more impressive than the differences. However, again there is labeling in III and IV and the run-off peak and no real elevation above background for the remaining histones. We then wished to examine the patterns of synthesis in the plant kingdom. We selected two systems: pea cotyledons and cultured tobacco cells. In order to avoid the problems of concomitant DNA synthesis we employed pea cotyledons from which the growing embryonic axis was cut off immediately prior to the experiment. The pea cotyledons were incubated in a sterile medium in the presence of antibiotics and C^{14}-leucine. The pattern of labeling found in this type of experiment is shown in Fig. 9. Again there is a slightly different optical pattern indicating slightly different histones. However, we see also the same general pattern found before; that is, a small amount of label in the run-off peak and in the III and IV peaks.

We had one more system which we could conveniently investigate. Here we had tobacco cells growing in exponential growth in a chemically defined medium. DNA synthesis continued apace. They were allowed to incorporate C^{14}-leucine to study the incorporation into all histone fractions. The pattern of histone biosynthesis is shown in Fig. 10. The next step was to take these cells and treat them with 5-FDU. We knew from the work of Birnstiel and Flamm (7) that in this system within two hours after a treatment with 10^{-6} *M* 5-FDU, we would totally inhibit DNA synthesis, without a serious reduction in RNA synthesis. We could now study a system where we had artificially inhibited DNA synthesis. We must bear in mind that the only thing we've done to alter the system is to impose a metabolic block to the formation of thymidine. Figure 11 shows the result of this treatment upon histone biosynthesis. There has been a change to the pattern observed in cells in which DNA synthesis was not normally being synthesized. Thus, it appears that by applying a

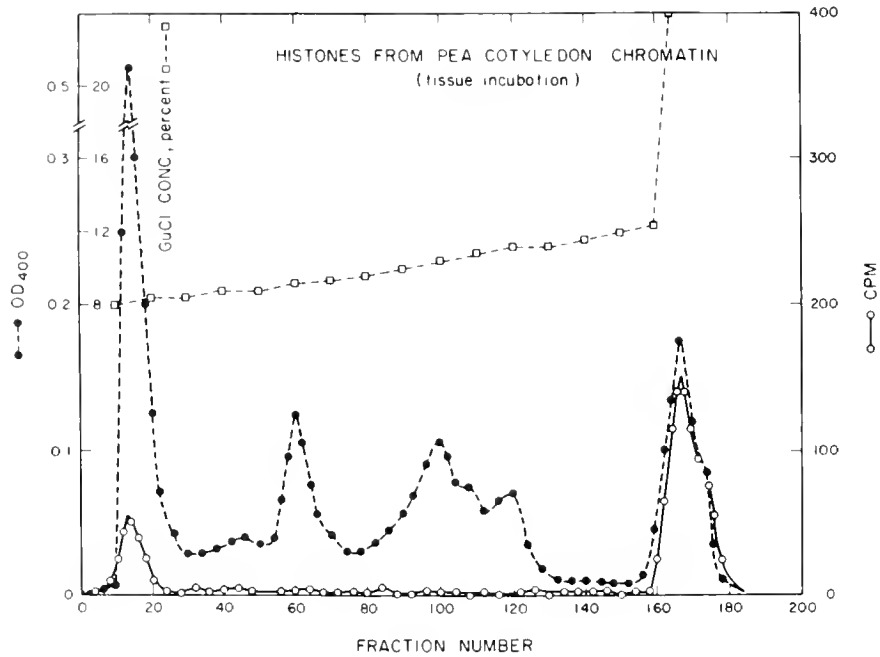


Fig. 9.

Biosynthesis of pea cotyledon histones. (Fig. 6, Chalkley and Maurer, *Proc. Natl. Acad. Sci. U.S. 54,* 498, 1965; reproduced with permission of the National Academy of Sciences.)

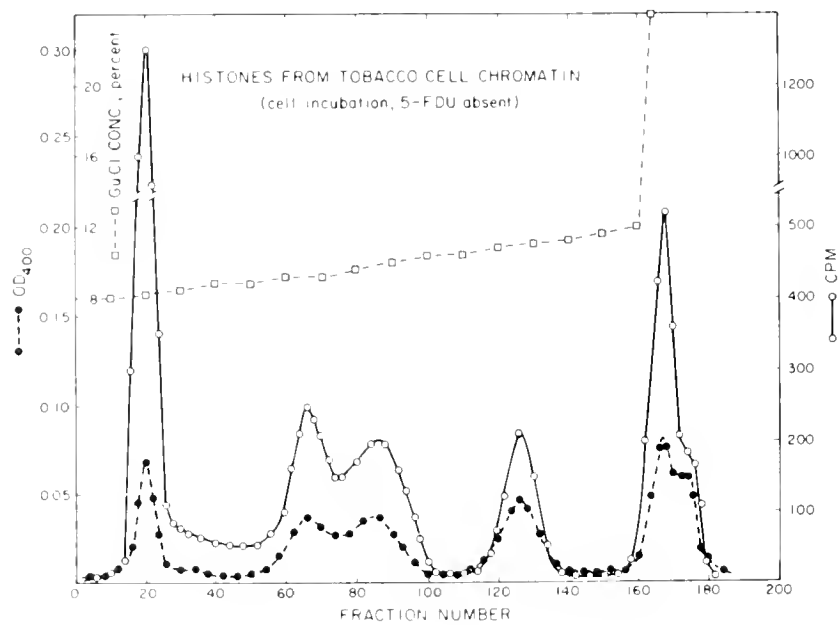


Fig. 10.

Biosynthesis of histones in cultured tobacco cells growing exponentially. (Fig. 8, Chalkley and Maurer, *Proc. Natl. Acad. Sci. U.S. 54,* 498, 1965; reproduced with permission of the National Academy of Sciences.)

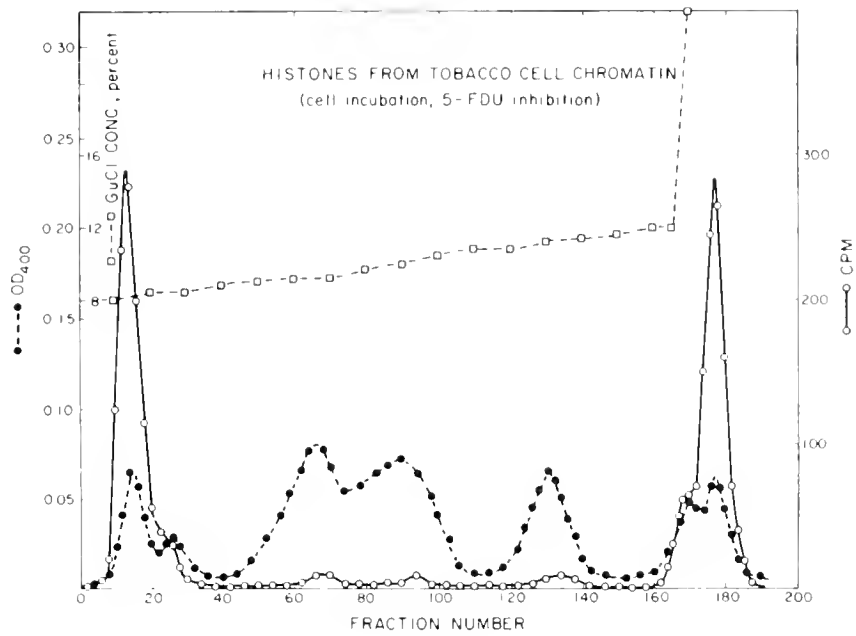


Fig. 11.

Biosynthesis of histones in cultured tobacco cells after inhibition of DNA synthesis with 5-FDU. (Fig. 7, Chalkley and Maurer, *Proc. Natl. Acad. Sci. U.S. 54*, 498, 1965; reproduced with permission of the National Academy of Sciences.)

very simple block to DNA replication, we are inhibiting the formation of certain types of histones.

A somewhat allied topic concerns the replication of DNA in the presence of histones. Although it has been suggested that histones might repress the function of RNA polymerase it is evident that many cells are quite capable of maintaining the function of DNA polymerase in the presence of this ubiquitous protein. Thus an *in vitro* study of the effect of DNA polymerase upon nucleohistone seemed an exciting realm for study. This has been pursued by Dr. S. Schwimmer. Of particular interest was the fate of the histone associated with the template. How would it distribute itself among the progeny molecules?

The plan of his experiments was somewhat similar to that adopted for the *in vitro* analysis of RNA synthesis. He isolated nucleohistone from calf thymus. This was incubated in the standard fashion for DNA synthesis. The products were examined on a free boundary electrophoresis apparatus (8) previously standardized relative to the electrophoretic mobilities of DNA and nucleohistone. The results are shown in Fig. 12. This shows that some of the radioactive precursor has been incorporated into material with the mobility of deproteinized DNA. In addition some radioactivity is seen in the region with the mobility expected for nucleohistone. An important question was to find out if the newly synthesized DNA had any histone associated with it. This was answered by exploiting the well known resistance of nucleohistone to DNase during a time period in which DNA alone is extensively degraded. Experiments showed that the newly synthesized material (measured in terms of cpm) is readily solubilized by DNase. Thus a rather curious result arises from these *in vitro* experiments, namely that the daughter strands of DNA are not associated with histone. If the parent molecule was in fact in such an association it is hard to explain this circumstance. So far there has been no resolution of this apparent inconsistency.

Evidence exists that the steroid hormones exert their primary effects at the genetic level and thus these hormones seem a useful tool with which to examine the molecular mechanisms of control in higher organisms. Some

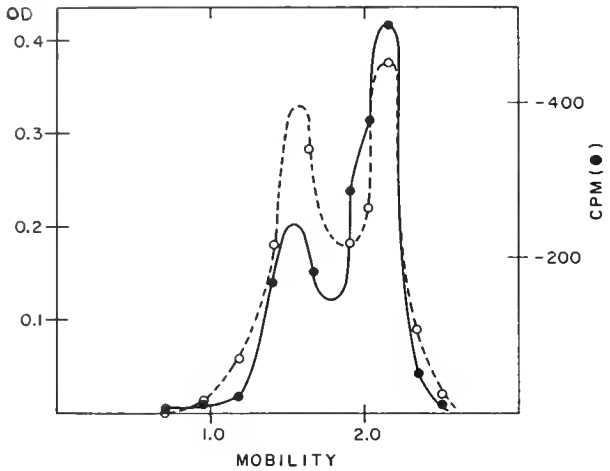


Fig. 12.

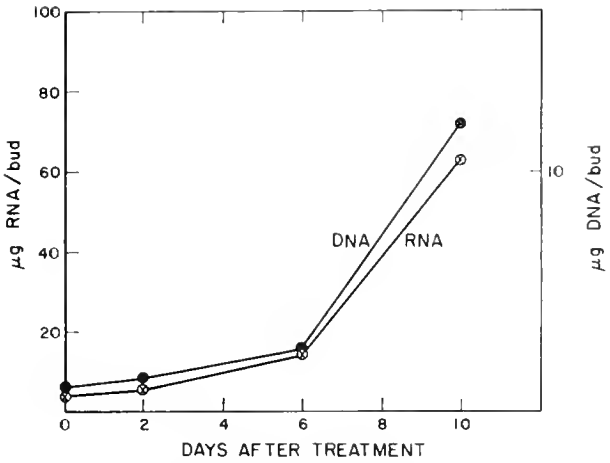
Free boundary electrophoresis of the product of nucleohistone-primed DNA synthesis. The faster component has the mobility of DNA (peak at 2.2), the slower has that of nucleohistone (peak at 1.6).

studies to this end have been initiated at Cal Tech. In particular I wish to discuss a number of experiments related to the release of dormancy in the potato tuber, to the increase in enzyme activity of the liver induced by hydrocortisone, and to the effects of estradiol in preparing the endometrial layer of the uterus for implantation following fertilization. The first experiments were performed by Dorothy Tuan (9). The system she has studied is the dormant bud in the potato tuber, the dormancy of which is relieved by ethylene chlorohydrin. Dormant buds of potato tubers are treated with ethylene chlorohydrin for three days and immediately there is an increase in DNA and RNA synthesis (Fig. 13). The RNA synthesis is actinomycin-D sensitive. If RNA synthesis is inhibited at this time in the development, DNA synthesis is also stopped. Therefore, the strategy of her next experiments with the system was to isolate chromatin from the buds at an early period where it is making very little RNA, and to compare its *in vitro* template activity with that of chromatin isolated from the buds at a later period in the development where RNA synthesis was much increased *in vivo*. Table IV shows the result of this type of investigation. Potato tuber chromatin was found to have an exceedingly low template activity. However, this is not necessarily significant since isolation of the chromatin from the tuber presents considerable technological difficulties due to the almost infinite amount of starch present. In the case of chromatin isolated from the dormant bud, we see that chromatin can direct the synthesis of RNA, but at a very low level. At the end of three days' treatment with ethylene chlorohydrin, the template activity of the bud chromatin is seen to increase. A significant increase in the amount of RNA synthesized is observed. Thus the template activity of isolated chromatin has mirrored its change in the pattern of RNA synthesis in development.

A similar approach has been applied to the study of the effect of hydrocortisone upon RNA synthesis in the liver of the rat. These studies have recently been reported by M. Dahmus and J. Bonner (10). The experimental design was to compare the template activity of liver chromatin adrenalectomized rats before and after hormone administration.

A characteristic result of this type of experiment is shown in Fig. 14. The template activity (rate of RNA synthesis) is plotted as a function of the increase in concentration of the DNA or purified chromatin in the incubation mixture. In the *in vivo* experiments the increase in RNA is some 300% (11). However, in this sort of study it is not nearly so dramatic. This may be due to a difficulty of getting some of the RNA in this system to leave the template. Due to the relatively small increase in chromatin template activity, statistical studies were applied (10) to this system and the difference shown to be significant at the 95% level. The possibility that an increase in RNA synthesis following hormone administration might be due to less RNase or ATPase in the *in vitro* system was checked and found not to be the case. The size of the DNA in the induced and noninduced chromatin was the same, as deduced from analytical ultracentrifuge studies. The deproteinized DNA from both types of chromatin were identical in their ability to direct DNA-dependent RNA synthesis.

In our experiments with estradiol we have adopted a different approach. Estradiol, applied *in vitro* to the endometrial cells of immature calves, stimulates RNA and protein synthesis followed by DNA synthesis and mitosis about 44 hours after the initial hormone application (12). The fact that it is possible to demonstrate such mitosis by microphotography (12) shows that the tissue incubated *in vitro* appears to be responding in the same way that the endometrial tissue is in the calf. One thing which interested us and which is pertinent to the problem of histones and the relationship of



RNA and DNA content of buds of potato tubers at varying times after 3-day pretreatment with ethylene chlorohydrin.

Fig. 13.

(Fig. IA, Tuan and Bonner, *Plant Physiol. 39*, 768, 1964; reproduced with the permission of the American Society of Plant Physiologists.)

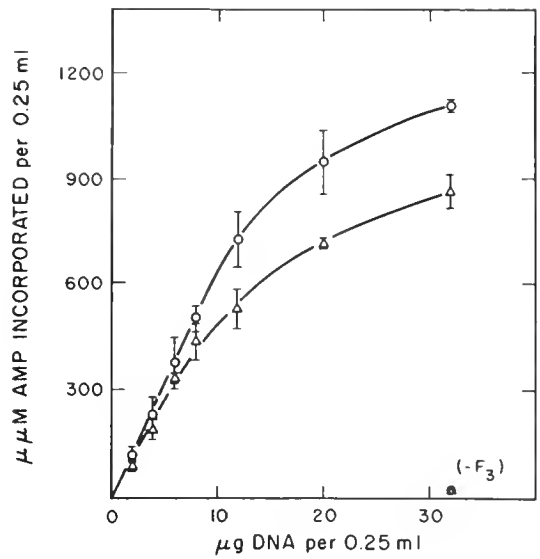


Fig. 14.

Template activity of rat liver chromatin isolated 4 hours after treatment with hydrocortisone (-o-) or saline (-△-).

histones to control was as follows. It has been reported recently in the literature that cells can be treated with hormones in such a way as to give a histone-hormone linkage, and it was implied that hormones might be pulling the histones off DNA. We had an excellent system with which to examine this hypothesis since we were able to study large amounts of target organ tissue.

We were anxious to see if endometrial tissue incubated *in vitro* followed some of the rules that one would expect from the *in vivo* endometrial material. Figure 15 gives an account of the uptake of hormones into the endometrial cell. There appears to be some degree of additional concentration of estradiol and progesterone. Progesterone is also a hormone which has the endometrium as a target tissue during pregnancy, and so it is not surprising that it is also concentrated into the tissue. Incorporation of hydrocortisone which, of course, has liver as its target organ was low. In Fig. 16 you see the uptake into the cytoplasmic fraction. It follows the overall pattern of the previous figure. However, now when we looked at the lysed nuclei [which I will refer to as crude chromatin (bottom, Fig. 16)] we began to see a very dramatic difference. Again, I stress that as yet I am discussing hormone uptake and not binding. There are large amounts present of the hormones for which

TABLE iV

Effectiveness of Chromatin of Dormant and of Non-dormant Potato Buds in the Support of DNA-dependent RNA Synthesis by Exogenous RNA Polymerase

For composition of reaction mixture see Materials and Methods.

50 μg of DNA supplied to system as:	RNA synthesized μμmole AMP incorp per 10 min
Potato DNA (deproteinized)	3370*
Chromatin of potato tuber	0
Chromatin of dormant buds	122
Chromatin of buds from tubers at end of 3-day treatment with ethylene chlorohydrin	1412
Chromatin of buds from tubers 10 days after 3-day treatment with ethylene chlorohydrin	1538

* Incorporation due to polymerase alone (150 μμmole) subtracted.

(Table I. Tuan and Bonner, *Plant Physiol. 39*, 768, 1964; reproduced with permission of the American Society of Plant Physiologists.)

this is the target tissue, and small amounts of the other steroids. I should add that the specific activity of testosterone and estradiol were within 2% of each other. Hydrocortisone was somewhat

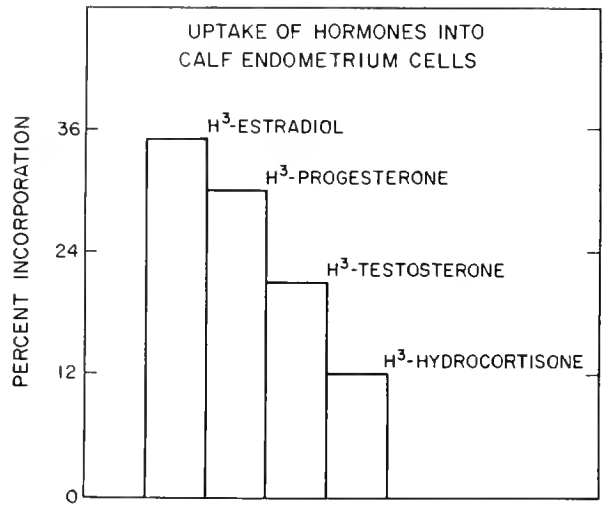


Fig. 15.

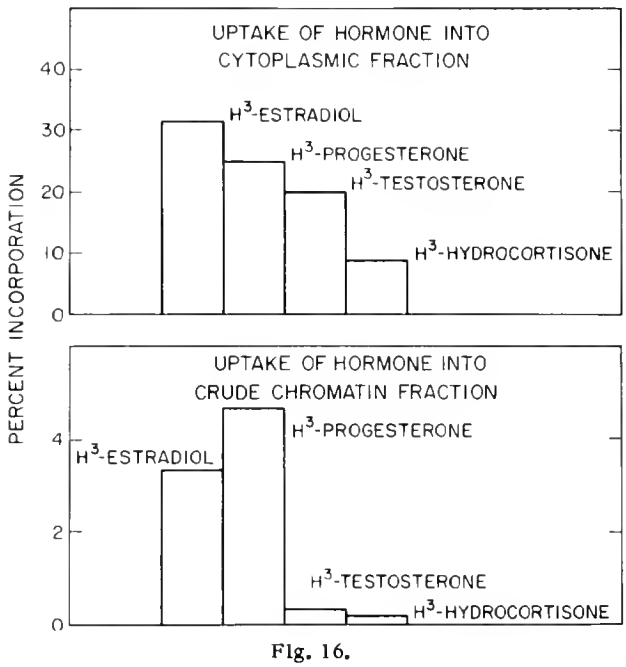


Fig. 16.

lower so it makes the interpretation of that result rather more difficult.

In the final figure of this trio we see the specific activity of hormone actually bound (fig. 17). I define "bound" as that hormone which can be centrifuged through a sucrose gradient along with the chromatin into the pellet and which isn't removed by subsequent exhaustive dialysis. Again, specific activity of the incorporation of the target tissue specific hormones is high and that of testosterone and hydrocortisone relatively low. More recent experiments demonstrated an even more dramatic effect with an equivalent technique.

GRUN: What tissue was this in?

CHALKLEY: This is the calf endometrium, the epithelial layer of the uterus, which had been scraped off and incubated.

SCHRAER: Do you analyze it for the hormone or just for the label?

CHALKLEY: Just for the label.*

SCHRAER: Do you know if it has hormones in those parts of the cells?

CHALKLEY: We put in labeled hormone and later on we follow the behavior of the counts. Now, it may be that this is being degraded and then the degraded material is being bound. So far we haven't found that out.

In Table V we see the effects of very different treatment on the chromat in containing the hormone. Organic solvents appear to reasonably efficiently extract a hormone. Sulfuric acid (2N) which we know will extract histones virtually quantitatively, also solubilized a portion of the counts. Guanidinium chloride, which we also know dissociates histones and denatures proteins, released over 50%. Sodium chloride (2 *M*) released only a small fraction of these counts. However, the real clue to the possibility of binding to histones was given, first of all, from histones isolated from this and put through the IRC-50 column. Not a single count above background was found and in fact essentially all of the hormone, after treatment with acid, was fully dialyzable. I should add, also that recently we've found that the hormone appears to be thermolabile. In 30 minutes at 37° about 50 to 60% of the hormone can be thermolabilized (13).

As a further check on what the binding really involved we suspended the chromatin in 2.09 *M* cesium chloride, which is of sufficiently high ionic strength to dissociate the histones and other chromosomal proteins, and then centrifuged the solution at high speed. You can actually band the histone component (14) (Fig. 18). The bulk of the non-histone protein bands at a lower density than the histone. This material aggregated as a very, very thin skin in the tube. A considerable number of the counts were localized in this skin which we homogenized and counted.

EPEL: Before you go into this new subject, could you clarify the conclusion from the estradiol experiment?

*Subsequent studies have demonstrated that 99% of the bound H^3 was present as unchanged estradiol-17β (13).

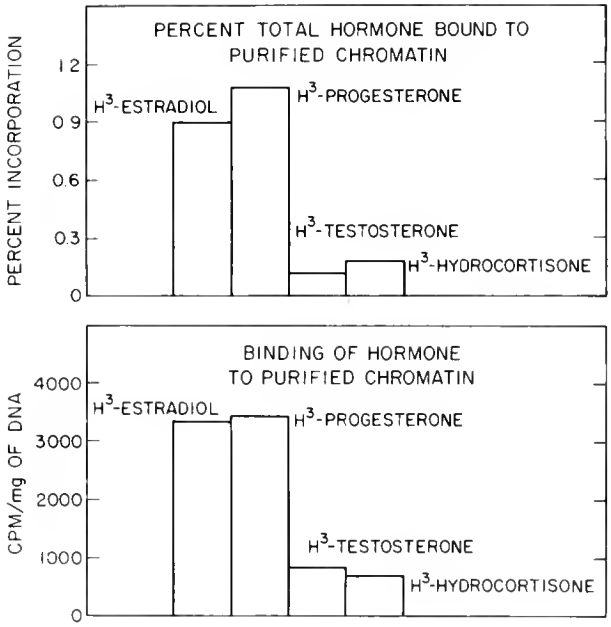


Fig. 17.

CHALKLEY: Well, the conclusion is that it appears to be bound to something which is lighter than histones. It's not bound, apparently, to any great extent to histones, as far as we can see. Possibly it's not bound to histone at all. It is bound to something which precipitates at this concentration of CsCl, and our efforts have been to try and isolate it further.

EPEL: Have you made any estimates of how many molecules of estradiol there are per nucleus?

CHALKLEY: No, we haven't yet.*

Well, now I want to think a little about the problems of repression and what we would have to require of any model to account for repression. We have to be able to explain differential gene effect, the problem of epigenetic control and differentiation. How is it that the pea cotyledon can synthesize globulin, and yet pea buds cannot synthesize any detectable amount of globulin? We have to involve in this model the fact that a substantial volume of histones does not turn over at all in the lifetime of a given DNA molecule. We have to explain the fact that some do turn over. We have to be able to explain induction of enzyme formation occurring at the genetic level. (This will have to account for hormonal induction). We have to demonstrate that if we induce a system and then remove the inducer that we have a reversal to repression. We have to demonstrate a gene specificity. This has always been a tremendous question mark with histone experiments. It has long been a problem to understand how to selectively repress a gene with simple electrostatic interactions.

*Recent studies suggest a value of about 2500 molecules of estradiol per nucleus.

TABLE V

Hormone Binding by Chromatin

Treatment	Per Cent Solubilised
EtOH	102
$CHCl_3$	89
Et_2O	92
0.2N H_2SO_4	65
2.3M GuCl	>50
0.15 NaCl	<10
2.0M NaCl	<10

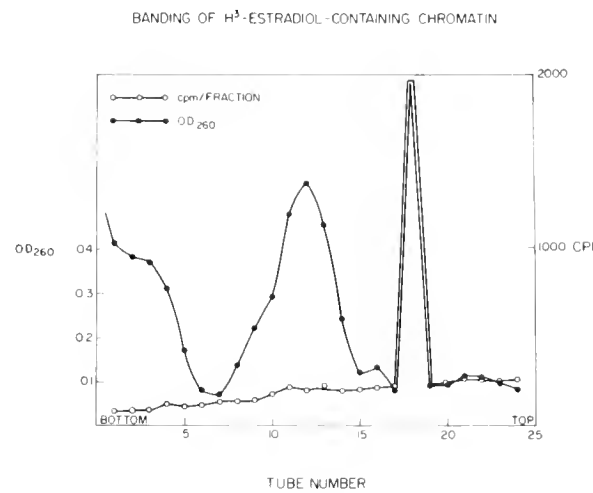


Fig. 18.

John Frenster, of the Rockefeller Institute, produced only recently in *Nature* (15) a model for the repression and specific gene action. What is proposed is that you have the whole genome entirely wrapped up with histones and repressed. He invokes histones as repressors. He then requires a specific derepressor of RNA that finds the section of the genome with which it is base complementary. It associates with

the DNA at this point and displaces histone. This leaves free just one strand to code for messenger RNA. That's fine, but I find it difficult to understand how this process is reversed; and also, it wasn't explained how he thought hormones would stimulate this removal process. Also he doesn't explain differential gene action or epigenetic control.

So, just for fun, Dr. Maurer and I present another model. It is shown in Fig. 19. This has at least the advantage, I think, of concentrating thought on a dynamic model rather than a static one, and I feel that it's a dynamic process that's involved in repression. Now, first of all, we have the genome of a differentiated tissue in which some specific areas of this genome are completely unavailable for genetic transcription. In the *in vitro* reconstitution experiments of Bonner and Huang it was found that the histones were virtually 100% efficient in cutting off DNA- dependent RNA synthesis. So, we have areas which are cut off with those histones; they're not allowed to be transcribed, and, during the lifetime of a particular DNA molecule, there will be no RNA synthesis in these genes. We have another basic protein; we haven't specified any more than to say it's basic, and based on the results of Huang and Bonner (14) we postualte a basic protein-RNA linkage. Now, if one wants to think of proteins linked to a very special RNA like this, it would seem to require a covalent linkage. Again we're speculating a little; we don't know that it is covalent, but the evidence is beginning to mount up toward that possibility. If that's so, these have to be linked, presumably, through a series of enzymic reactions. This material which we call a functional repressor has the ability to recognize a specific gene because of this RNA. It is then possible to conceive of a dynamic equilibrium in a section of the genome. We suggest thinking along the lines of some sort of dynamic equilibrium with the repressor going on and the repressor falling off and being degraded. Now, if we postulate this dynamic system, then it's possible to conceive of induction acting by somehow inhibiting the linkage of these two component parts in the formation of the functional repressor. In this case, the repressor is not formed, so the equilibrium will shift away from the genome. Protein synthesis malfunction would also give rise to a decrease in the concentration of the repressor, as would inhibition of RNA synthesis. Perhaps we can begin the discussion with this model that we have proposed.

GROSS: How do you get the stable histones which are not turning over? How is their specificity compared to the one which does?

CHALKELY: Their synthesis would require both spatial and apparently temporal specificity - whether this synthesis is directed by messenger RNA or by other methods is an interesting problem.

DYNAMIC MODEL FOR INDUCTION AND REPRESSION

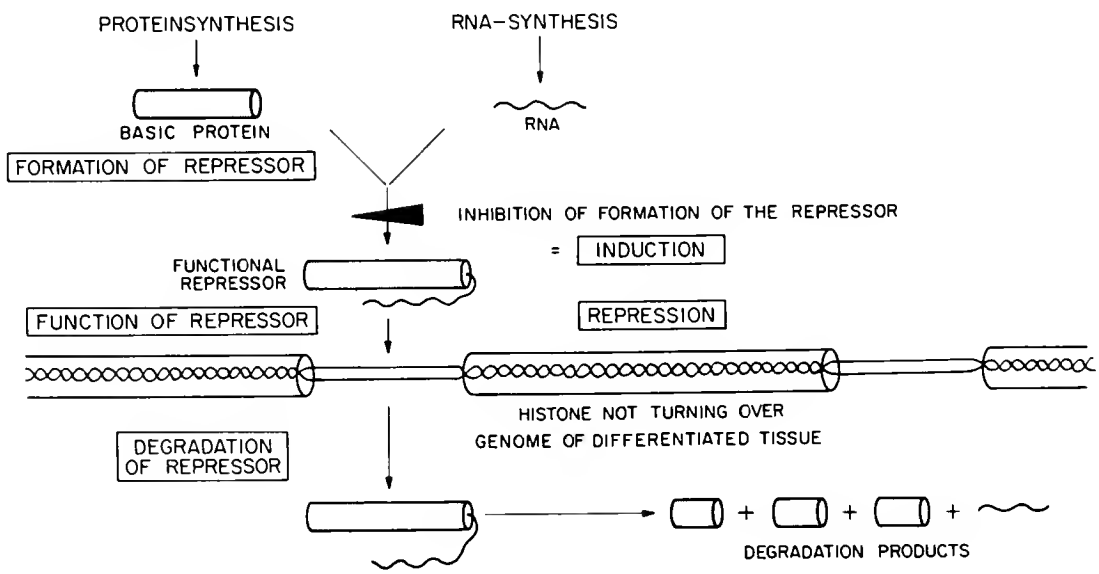


Fig. 19.

GROSS: It imposes an analytical requirement on the model. If you're going to have recognition, then you have to have sufficient length of RNA to recognize the gene or the cistron.

CHALKLEY: How big is an operator gene?

GROSS: Well, it's a fraction or a few per cent of an operon. However, what I'm suggesting is that isolated chromatin ought to have a lot of RNA.

CHALKLEY: It has some RNA. When you isolate chromatin and nucleohistone it has not got a great deal of RNA; nonetheless a residual quantity of RNA is always found. This RNA is very difficult to remove. It's resistant to RNase, unless you pre-treat it with DNase or unless you heat it to 60°.

KAHN: I'm curious. Where do you postulate that the RNA for the functional repressors is synthesized? It seems to me that the most likely spot would be the very same portion of the DNA template that it will later repress.

CHALKLEY: I think that is, in fact, very reasonable.

PERSON: Are you going to have repression by the RNA or by the protein?

CHALKLEY: I imagine it's the protein that's involved in the repression. So far it has been shown in our group that you can put cationic polypeptides on the template and get a repression of the DNA-dependent RNA synthesis. It's a fact you can work on anyhow.

TS'O: I would like to make a statement about this. The most difficult problem in setting up the hypothesis for histone or any proteins to be the genetic repressor is that we know nothing about how the proteins and the nucleic acids interact specifically. How do proteins recognize the base sequences or the base composition of the nucleic acids? The present status of our biochemical knowledge does not give any model in molecular terms which would lead to this kind of specificity. For instance, in protein synthesis, even though the transfer RNA can form base pairs with the messenger RNA, the translation of the genetic codes is dependent upon the recognition process between the transfer RNA and the amino acid activating enzyme. What you are trying to do here is almost the same thing, because you are trying to use the RNA to recognize the DNA through the accepted based pairing mechanism. However, specific base proteins have to be able to recognize and be attached to the specific RNA.

CHALKLEY: Now you're putting a requirement in which I don't think is necessary. All we have to say is that there has to be an enzyme or enzymes to link those two and this enzyme has to be able to recognize the basic protein. Now, surely one protein can recognize another protein?

POLLARD: I feel you're missing one very important question. I think your model is very good, but there has to be something that really rips the RNA off. It can't just come off; it has to be torn off. Now, if you were to take just the soluble RNA and quietly put histone on the end of the RNA, you would block the tear-off mechanism. The rate at which that stuff comes off is impressive. It's linked in complementary links of base pairing. Nevertheless, it comes off. No one has been able to observe the time it takes.

CHALKLEY: That's true.

POLLARD: Also, if you were going to have as small a section of the DNA in the bacterial cell as you indicated, then the length of the RNA being torn off must be 15 times as long.

CHALKLEY: Yes, this was an arbitrary length for purposes of discussion.

POLLARD: I suspect you may be on the right track with this, but the RNA just doesn't do anything you like.

MAURER: It should be pointed out that this is a kind of equilibrium and therefore it is sensitive to all kinds of factors which influence this equilibrium. If one enhances the degradation, for example, it would certainly have an effect on it, probably by shifting the rate of reactions. Furthermore, I would like to stress, in respect to the hormone studies so far done, that it isn't yet completely clear whether there is an RNA polymerase increase due to new formation of the RNA polymerase or to an activation of the enzyme as far as RNA template synthesis is concerned. However, in these experiments which Dr. Chalkley has presented, it was shown that there is an increase in template activity since we put in high excess of RNA polymerase. This is the main point. It really looks like the hormones are, by some means, removing some kind of repressor and not just increasing the RNA polymerase. We suggest that hormones inhibit the formation of some functional repressor and in this way alter the dynamics.

PERSON: Wouldn't you than want them connected to the things that are being permanently repressed in the model, so that they could open up new regions?

CHALKLEY: Well, if you do that, you have to specifically involve proteins other than histones in this sort of specific permanent gene repression and this may be quite possible. There

may well be proteins other than histones involved, but I was basing it on the results in which we've come to the conclusion that the hormone hasn't got as one of its functions to bind histone, in any sort of binding we could detect. So, one of the original reasons we developed this idea was because we had to explain an increase in template activity. So, we had to postulate some sort of a dynamic equilibrium and determine if we could interfere, not by reaction with histone, but by reaction with another protein. The suggestion here is that the protein interacts with a hormone.

The point I really wanted to make and really wanted to stress is that we would like to approach the problem in terms of a dynamic situation and then see if we can disturb the equilibrium in some way at some point; and this may give rise to derepression. If you remove the inhibitor, everything flows back and you set up the original equilibrium.

EPEL: I'd like to try to integrate your thinking with Dr. Wright's stimulating paper this morning. In your evidence regarding the hormonal action you claim that 95% of the hormone is in the cytoplasm - is that right?

CHALKLEY: We've found large percentages in the cytoplasm but not large percentages *bound* in the cytoplasm.

EPEL: Well, here is what I want to bring out. In our discussion here, we know that these hormones, such as estradiol or leutenizing hormones, can fantastically affect intermediary metabolism. These are very fast effects. Let's take ACTH in the adrenal cortex; it will activate cyclic AMP formation and thence glycogen phosphorylase. There are also a number of papers showing direct cyclic AMP stimulation of enzymes involved in steroid synthesis and there is, also, activation of protein synthesis via pre-existing messenger RNA. So, isn't it conceivable that you have a system here in which numerous changes are triggered as a natural consequence of the hormonal action, not through the hormone directly but through the changing levels of various intermediates? This, then could act on the nucleus?

CHALKLEY: Yes, I would say that is entirely possible.

EPEL: I'm just trying to integrate these two very interesting lines of reasoning.

CHALKLEY: Well, some of these hormones, of course, repress and activate preexisting enzymes. This is well catalogued; and we know that they bind slightly into the cytoplasmic fraction. But on the other hand there is a body of evidence available which suggests that an early biochemical effect of hormone administration is increased RNA and protein synthesis. It is entirely possible that the nuclear binding and the increased RNA synthesis are related.

EPEL: The hormone can be many steps removed from its final action. It's at least 3 or 4 steps back, for instance, in initiating cyclic AMP formation.

MAURER: Yes, but why do we always find a lot of hormone in the nuclei? In the case of ecdysone, for example, we find hormone about 20 minutes after injection into the larvae of *Calliphora erythrocephala* in the nuclei; and the earliest time it's been found is about 10 minutes after injection. However, you are quite right; it's still not ruled out that there isn't some kind of reaction with the cytoplasm.

CHALKLEY: There is one thing I possibly didn't bring out fully enough. If we do this hormone incorporation experiment, a great deal of the hormone goes into the cytoplasm, but if you look at the amount *bound* there is a much greater degree of binding occurring in the nucleus.

EPEL: Are these experiments in the range of physiological concentration? Perhaps the experiment to do would be to use an extremely small amount of hormone, and then see if it appears in the nucleus.

MAURER: Actually this has been done with physiological 10^{-6} *M* concentration. So, it's conceivable although not completely proved.

GROSS: What were you saying about ecdysone? When we studied this system, although it is binding to the ribosomal particles, the amount of binding in the nucleus is not substantial.

CHALKLEY: I was recalling the results with binding in the mitochondrial and ribosomal fractions, because it has been shown that binding occurs in these particular fractions.

GROSS: Where does the hormone exert its first recognizable characteristic effect? In this case isn't the first characteristic response in the genome?

MAURER: I treated the insect larvae with ecdysone and I found the highest activity in the nuclei and not in the mitochondria. I found some activity in the mitochondria and even in the microsomes, but the highest activity after one hours was in the nuclei. This was then followed by a decreasing level; so it looks suspiciously as though the hormone first migrates into the nuclei and then by some process is depleted.

SCHRAER: You keep using the word hormone. Do you mean labeled hormone?

CHALKLEY: Yes.

KOHNE: Could there be membrane material in this chromatin preparation? There have been reports that even purified DNA has some membrane in it.

CHALKLEY: We've never treated with deoxycholine. This might be worth doing, though I suspect it would dissociate some histone from the DNA.

PAPACONSTANTINOUS: You know, there's one question that hasn't come up yet, and I don't know that there's any answer. That is, how does your system fit in with the question "Is the genome read when the cell is replicating or is it not read?" If the genome isn't read during replication, then you've got to fit up all your repressors for replication and then pull them back off again.

CHALKLEY: I don't think I have an answer to that.

PAPACONSTANTINOU: Well, I don't know whether there is an answer to that very question of whether it's being read or not.

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DYNAMICS OF THE POINT OF NO RETURN DURING DIFFERENTIATION IN *BLASTOCLADIELLA EMERSONII*

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East Lansing, Michigan

I left East Lansing a few days ago knowing that cell differentiation and morphogenesis were riddles which had served admirably for many years as focal points for honorable speculation. I will leave Penn State, today, with the strong suspicion that solutions to these problems are far from just around the corner. I trust, therefore, that I will be forgiven if, during the short time we have left this morning, I add some haze of my own to this generally smoggy area.

Almost twenty years ago, in a fresh-water pond behind old MacFarlane Hall on the campus of the University of Pennsylvania, I discovered, isolated in pure culture, and subsequently christened as a new species, the Phycomycete known as *Blastocladiella emersonii* I put this specific epithet upon it because of fond memories of my first real teacher, Professor Ralph Emerson, who but a few years before had introduced me to the fascinating antics of these ubiquitous aquatic fungi commonly known among mycologists as the water molds.

I shall spend perhaps half of my time, this morning, developing *Blastocladiella's* background. This will serve a dual purpose, because Dr. Lovett, who follows me on the program, will also be discussing his studies of *B. emersonii*. Let me begin, therefore, by showing you more or less what I saw in 1948 when I took the first spore of *B. emersonii* ever to be rendered captive and put it on a slab of nutrient agar medium in a Petri dish (Fig. 1). The spore germinated and developed into a little plant with root-like rhizoids. At maturity, almost (but not quite) all of this thallus was converted into a spore-bearing sac, the sporangium. The first figure shows a cross section through a spore sac in which the protoplast has been cleaved up into spores. Subsequently, these spores were liberated through exit pores in the sporangial wall and settled on the surface of the agar immediately around the parent plant. The latter, thus depleted of its protoplasm and now an empty shell, collapsed. The newly liberated spores, however,

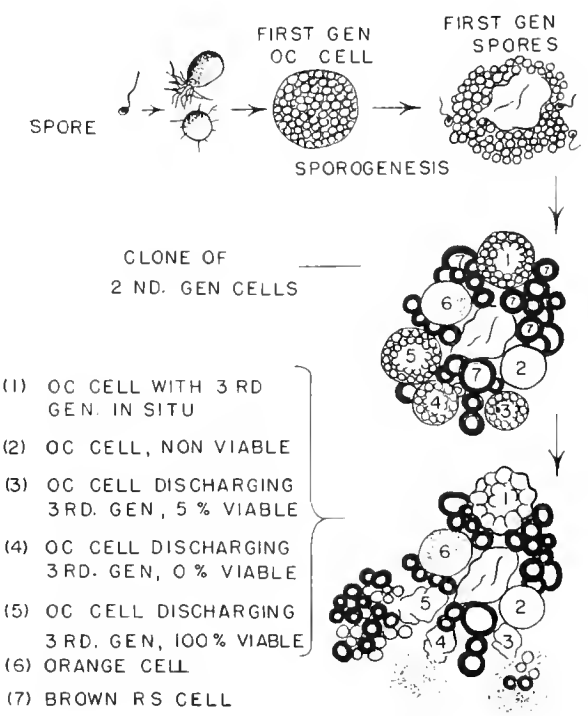


Fig. 1.

Schematic representation of the totipotency of 2nd and 3rd generation clones derived from a single spore of *Blastocladiella emersonii*.

began to develop into a clone of second generation *Blastocladiellas*, and therein the totipotency of this organism began to manifest itself. There appeared, gradually, big cells and small cells, brown, colorless and orange cells, cells with thick walls - some sculptured and some not - and cells with thin ones, dead cells and living cells, cells which discharged yet another generation of spores among which no spores were viable, or in which some or all were viable. This, then, will suffice to show the general nature of the problem as it appeared at the outset in the late 1940's.

During the past two decades, some -but by no means all- aspects of this picture have been unscrambled and clarified. Let us deal with one of these, now, by way of Fig. 2. We know that the spore with which *B. emersonii* begins its life history is motile, uniflagellate, uninucleate and organized in a very crafty and intriguing fashion; its internal structure is unique. Indeed, its architecture has a direct and important bearing upon the story I will try to develop. However, inasmuch as Dr. Lovett will devote all of his talk to the spore and its activities, I will by-pass it without further comment. In synchronized single generation cultures, some 99% of the germlings of *B. emersonii* can develop along either of two major morphogenetic pathways. Along both of these paths, the thallus increases at an exponential rate in dry weight, volume and other features. A point is reached, however,

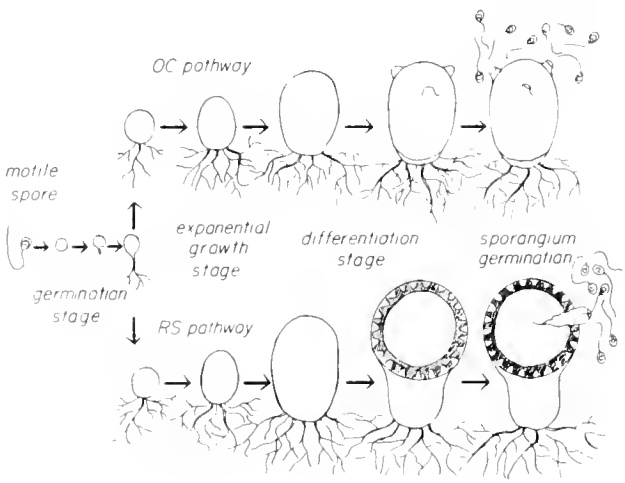


Fig. 2.

The two major developmental pathways taken by *Blastocladiella emersonii*. (Fig. 2, Cantino and Lovett, *In* "Adv. in Morphogenesis," III, 1964; reproduced with permission of Academic Press.)

when this exponential phase in development ceases; at this point, the plant embarks upon its second developmental stage, i.e., cell differentiation. The first visible evidence that this is about to happen is the formation of the septum, which delimits the thallus into a large upper cell and a small lower cell with root-like rhizoidal appendages. At this point, it is important to call attention to the fact that at maturity, the lower cell is (as far as is known) devoid of contents. The upper cell possesses all the cytoplasm and all the nuclei - hundreds to thousands of them, depending upon the environmental conditions selected - which are embedded therein. Thus, it should be recognized at the start that we are dealing here with the development of a *single cell*, for we are starting with a uninucleate spore and ending with a multinucleate coenocyte. Finally, however, at the very end of this organism's life history, these many nuclei embedded in a common cytoplasm do become delimited from one another by the formation of cross walls - or, perhaps more appropriately, cross "membranes." In any case, each nucleus functions as a focal point for the formation of a spore. Each nucleus inherits a tail, a mitochondrion and a number of other organelles, and the lot becomes surrounded by the flexible spore wall. This population of nuclei is then liberated from the parent cell in the form of a population of spores, and we are back where we began.

Now, let me retrace my steps for just one moment. Long ago, we labeled the uppermost cell in the top pathway (Fig. 2) an ordinary colorless cell, or an "OC" cell for short. The uppermost cell in the lower pathway is a resistant sporangial cell, or an "RS" cell for short. These two cell types differ from one another in many ways, some very obvious but others deceptively subtle. The more obvious differences include the following: the OC cell wall is chitinous and thin, while the RS cell wall, also chitinous, is much thicker; the OC cell wall is essentially colorless, while the RS cell wall is brown, being impregnated with melanin. The protoplast of the OC cell contains no detectable colored carotenoids, while that of the RS cell contains gamma-carotene. There are many, many other differences, but these are too numerous to mention now.

Finally, we also learned that these developmental pathways could be controlled at will by the simple expedient of providing the organism with a little bit of baking soda. In the presence of a suitable amount of exogenous bicarbonate, essentially all spores developed along the RS

pathway; in the absence of bicarbonate, they developed along the OC path. Thus, when this work began, we set out to learn by what means this bicarbonate trigger mechanism brought about the *de novo* synthesis of melanin and carotene, the increased rate of synthesis of chitin, and the numerous other characteristics associated with the structure and function of the resistant sporangial cell.

In the late 1950's, mainly through the skillful and conscientious efforts of my former students - Drs. James Lovett, Howard McCurdy, Evelyn Horenstein and others - methods were envolved for growing submerged, uniformly distributed cells of the fungus along one pathway or the other in massive, synchronized, single-generation cultures. By starting with several hundred million or a billion spores, all of them of exactly known age, such spores were made to go through their acts in nearly perfect synchrony along either path. Thus, it becomes immediately evident that, by this means, one can harvest almost any number of OC cells all of which have arrived at, let us say, 30% of their exponential period of growth; or OC cells all of which are tooling up to manufacture a second generation of spores; or a billion RS cells all of which are in the process of laying down the cross wall which will delimit the resistant sporangium from the rest of the thallus, and so on. Here, then, was an elegant system for studying the relations between biochemical and morphological differentiation, and we began to exploit it.

TILL: Have you got the time scale on this?

CANTINO: The time scale for the OC pathway depends upon the nature of the medium and other factors. Under the conditions we have used, it ranges from 12 to 17 hours, depending on population density. For the RS pathway, our standard procedure yields a generation time of 84 hours.

TILL: The other point I'd like to ask about is, are those spores the same?

CANTINO: Morphologically, they appear to be identical. (Note added in proofs: It should be pointed out that all of our work with synchronized liquid cultures is done with spores derived from OC cells. The reason for this is that it is a simple matter to prepare massive suspension of such spores, but a much more difficult one to prepare them from RS cells. There is some evidence, however, that the developmental potentials of - i.e., the kinds of progeny produced by - RS-spores and OC-spores are not identical (1)).

Now, let us come to grips with the nature of this bicarbonate trigger mechanism. This has been under investigation in my laboratory for a long time. Therefore, let me simply present a condensed recapitulation of the essential biochemical event which we think is operative; then, we can use this as a convenient point of departure. At the metabolic level, the focal point appears to be as follows. In homogenates of OC cells of various ages, the enzymatic activities of the tricarboxylic acid cycle are detectable; thus, the cycle is at least potentially functional. In spores and germlings of such OC cells, the *in vivo* evidence suggests that the Krebs cycle is operating. In Fig. 3, on the left, only one of the steps in the cycle is shown - the step mediated by a TPN-specific isocitric dehydrogenase. But, when bicarbonate is added to a spore or developing germling, it quickly induces a multiple set of enzymatic lesions in the tricarboxylic acid cycle. However, the isocitric dehydrogenase remains functional, and it now begins to operate in reverse, mediating reductive carboxylation of ketoglutarate back to isocitrate (Fig. 3, right). At the same time, bicarbonate also induces the formation of isocitratase, which cleaves the isocitrate to succinate and glyoxylate and thus prevents its accumulation. Finally, a constitutive glycine-alanine transaminase in the organism helps to keep the chain of reactions on the move by amination of glyoxylate to glycine at the expense of alanine.

Let me illustrate, by way of a few examples, some of the kinds of information obtainable

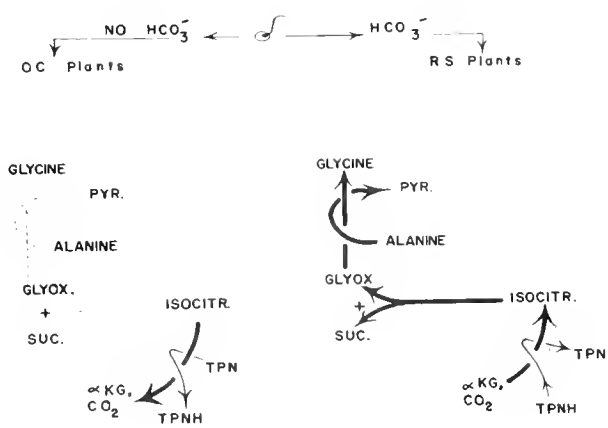


Fig. 3.

The bicarbonate trigger mechanism in *Blastocladiella emersonii*. (Fig. 6, Cantino, *In* "11th Symp. of the Soc. for Gen. Microbiol.", 1961; reproduced with permission of the Society for General Microbiology.)

with - and, I think, *only* obtainable with - synchronized single generation cultures, which support this general picture that I have been trying to create.

One of the first changes induced by bicarbonate, and detectable *in vivo* immediately after spore germination, has to do with gas exchange (Fig. 4). The upper curve reveals the course of oxygen consumption by an OC cell growing in the absence of bicarbonate. The lower curve shows what happens when bicarbonate is present in the medium. After the spore has germinated, there occurs an immediate and precipitous drop in oxygen consumption. Simultaneously, the exponential growth rate is reduced to 46% of that of the cell growing in the absence of bicarbonate. Also, bicarbonate causes the exponential rate of synthesis of the cell's pool of a soluble polysaccharide (made up solely of glucose) to double relative to the cell's exponential rate of growth in mass. These facts do not, of course, prove that lesions have developed in the tricarboxylic acid cycle as outlined above; they are, however, consistent with this interpretation.

The available quantitative data which deal with the enzymes themselves have a direct bearing at this point in our discussion. For example, cells growing along both developmental pathways have been assayed at various stages in ontogeny for isocitric dehydrogenase and ketoglutaric dehydrogenase activities. When the data are plotted, not as specific activities but rather as total units of enzyme activity per cell, it turns out that the exponential rate at which isocitric dehydrogenase accumulates in the cell during its exponential growth phase is about seven times higher than the rate at which the ketoglutaric dehydrogenase complex does so (Fig. 5). Furthermore, as will be seen shortly, the net accumulation of this latter enzyme system in the cell levels off and ceases long before that of isocitric dehydrogenase. What does this observation have to do with the question of oxygen consumption? A glance back at the previous slide will show that during the exponential growth of a developing RS cell, oxygen consumption decreases to about one-tenth of its starting level (Q_{O_2} = *ca.* 100) in the spore. If oxygen consumption by the growing cell were totally and exclusively dependent upon the operation of the tricarboxylic acid cycle, one might expect that the rate of turnover of the cycle would also drop to one-tenth of its starting rate at zero time. The quantitative data associated with Fig. 5 are consistent with this thought. From spore stage to end of exponential growth, the total units of isocitric dehydrogenase per cell increase 6,500 times, but the total units of

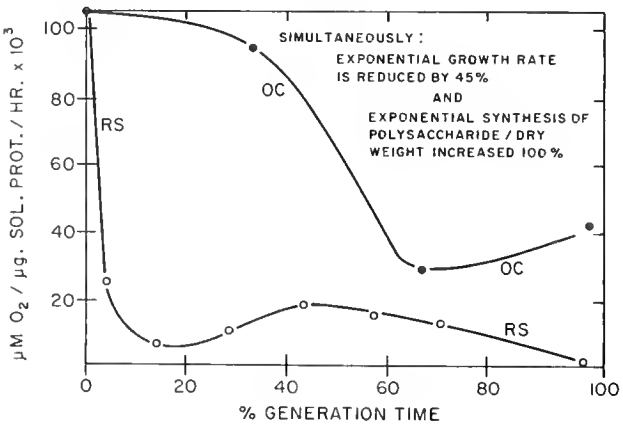


Fig. 4.

Oxygen consumption by OC and RS cells.

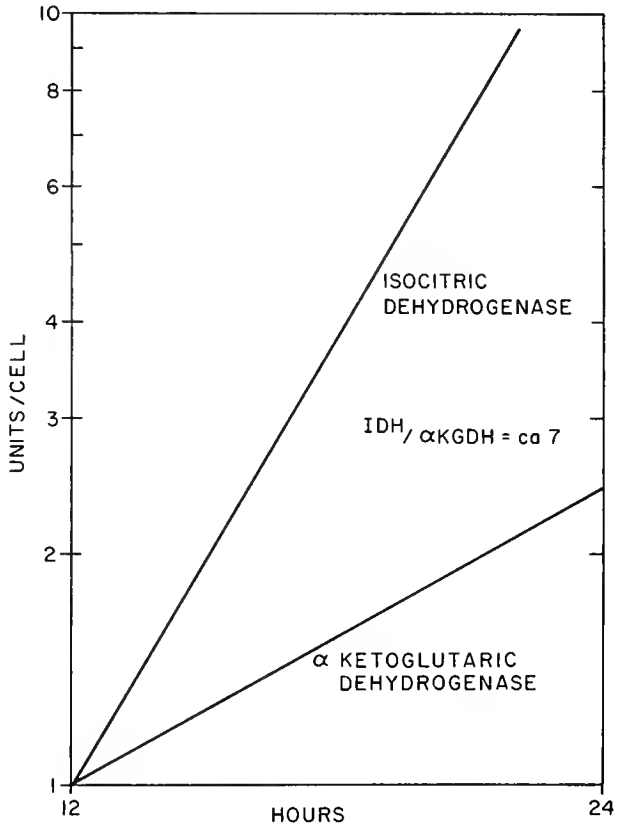


Fig. 5.

A comparison of the exponential rates of synthesis of isocitric and α-ketoglutaric dehydrogenase per RS cell during exponential growth.

ketoglutaric dehydrogenase increase only 650 times! Thus, the 90% decrease in oxygen consumption goes hand in hand with the 90% decrease in the intracellular accumulation of ketoglutaric dehydrogenase relative to the isocitric dehydrogenase which immediately precedes it in the Krebs cycle. It appears, therefore, as if bicarbonate causes the ketoglutaric dehydrogenase system to become a bottleneck in the cycle, that it begins to do so early in ontogeny, and that this soon brings the activity of the tricarboxylic acid cycle to a halt.

What about the other critical enzyme in the scheme - the isocitratase? Some years ago, Dr. McCurdy purified it and established its properties. Assays with synchronized cultures show how it, too, is involved. Figure 6 reveals what happens to the total units of isocitratase per cell during development in the presence and absence of bicarbonate. The intracellular quantity of this enzyme in the spore is shown on the vertical axis, i.e., at zero time. As the spore gives rise to a germling, and it in turn develops exponentially into a young OC plant in the absence of bicarbonate (bottom curve), there is no net synthesis of this enzyme. It seems as if the original amount of isocitratase in the spore is simply diluted out as the growing cell increases in size. Only when the OC cell has reached about half of its generation time does synthesis of isocitratase begin. However, when spores are germinated in bicarbonate media, exponential synthesis of isocitratase apparently begins immediately (upper curve). In summary, while bicarbonate brings about a lesion in the tricarboxylic acid cycle by creating a bottleneck at the locus of ketoglutaric dehydrogenase, it provides relief for the damage done by inducing, simultaneously, synthesis of isocitratase. Thus, in the bicarbonate-induced RS cell, isocitrate leads to succinate and glyoxylate (and thence to glycine), whereas in the bicarbonate-independent OC cell, it leads to ketoglutarate (and thence to succinate) and CO_2.

Finally, let me present one last set of data which bear upon this mechanism. If the proposed scheme is correct, *in vivo* uptake of CO_2 and/or bicarbonate by a developing RS cell should reach its peak at that point in ontogeny where the cell's complement of isocitric dehydrogenase is maximum relative to its complement of the bottleneck enzyme, ketoglutaric dehydrogenase (this point in ontogeny, as will be seen in a subsequent figure, occurs at about 36 hours, i.e., 43% of the RS cell's generation time). To test this notion, RS cells were grown in synchronized culture and then, at 30 hours, provided with a dose of $H^{14}CO_3^-$ and allowed to continue growing for 6 hours. During this latter period, cells were sampled and assayed for total ^{14}C fixed, and the medium assayed for total ^{14}C which had disappeared. A similar experiment was done in which 38 hour cells were fed the $H^{14}CO_3^-$. The results were combined to yield one graphs as shown in Fig. 7. You will note that uptake of $H^{14}CO_3^-$ per cell increases as the cell passes through 36 hours of age, and that uptake decreases again after 39-40 hours. Since the ratio of total units of ioscitric dehydrogenase to units of ketoglutaric dehydrogenase is maximum at 36 hours, and then decreases once again beyond this point, these data provide further evidence that the bicarbonate trigger mechanism operates as proposed above.

Let us move on, now, to consider the transition period between exponential growth of the RS cell and its subsequent differentiation; the data I wish to present in this connection also have a direct bearing upon the biochemical mechanism we have been discussing. The photographs which are shown in Fig. 8 were taken by Dr. Lovett when he was working in my lab,

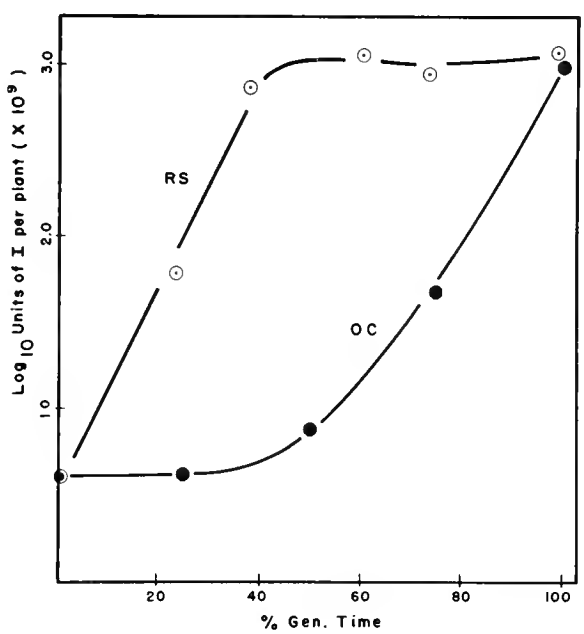


Fig. 6.

Synthesis of isocitratase during growth of OC and RS cells. (Fig. 5, Cantino, *In* "11th Symp. of the Soc. for Gen. Microbiol.," 1961; reproduced with permission of the Society for General Microbiology.)

and they reveal the microscopic appearance of RS cells at various stages in their development in a synchronized culture. During ontogeny, a point is reached beyond which the cell becomes irreversibly committed to RS formation. This is the morphogenetic point of no return. It is figuratively represented by the point of dichotomy of the two arrows, and it amounts to 43% of the RS generation time - chronologically, 36 hours under the conditions we use for growth. Before this point is reached, the cell's morphological potential displays an inherent plasticity; if the bicarbonate is removed from its environment, it reverses "direction" and embarks upon the alternate morphogenetic pathway. In other words, functionally it turns into an OC cell. However, beyond this point of no return, removal of bicarbonate does not cause morphogenetic reversal; the cell continues on its way toward the RS type whether or not the bicarbonate is still present.

This feature further emphasizes the fact that a synchronized culture of *Blastocladiella emersonii* represents an easily exploitable system for experimental studies of morphogenesis. Indeed, it is for this reason that I and my associates, past and present, have been trying to track the various events - intracellular and extracellular - which are associated with the genesis of a resistant sporangium as it approaches, passes through and then departs again from its point of no return. Many of these events have been followed on a per-cell basis, and a superficial digest of the results is seen in Fig. 9. You will note that many things begin to increase at an exponential rate - albeit not all of them at identical rates - after spore germination, and cease to do so at the point of no return. Others, glucose uptake for example, begin much later but still end at this same point of no return. However, other features, such as weight, lipid, total nitrogen, chitin, polysaccharide, RNA, melanin, etc., continue to increase to different stages in ontogeny beyond the point of no return. Then again, there are still other events which commence only at or beyond the point of no return. Clearly, then, it was of interest to find out which, if any, of the qualities associated with an RS cell before its point of no return would change to a new state more characteristic of an OC cell if morphological reversal were induced.

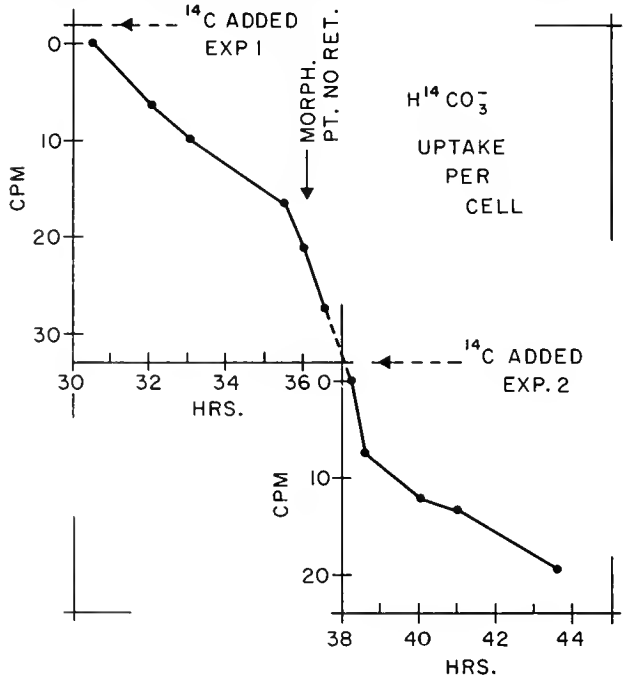


Fig. 7.

Uptake of $H^{14}CO_3^-$ by an RS cell as it approaches and passes the morphological point of no return.

We have done tests of this sort, and I would like to show you the results that were obtained from one such experiment that Dr. Lovett and I did some years ago. Figure 10 shows what happens to isocitric dehydrogenase and ketoglutaric dehydrogenase during development along the RS path up to a stage well beyond the point of no return. For convenience in making comparisons, the total units per cell at the spore stage were set at one for both enzymes, and all other values were then related to this and plotted accordingly. When bicarbonate was removed from RS cells a few hours before the point of no return, thus inducing morphological reversal, the total units/cell of isocitric dehydrogenase dropped sharply (whereas without reversal, it continued to rise) and the total units/cell of ketoglutaric dehydrogenase rose sharply (whereas without reversal, it did not do so). These results are represented by the dotted lines in Fig. 10. When this same kind of experiment is done with cells which have gone beyond the point of no return - and which, therefore, have lost the capacity for morphological reversal - the total units/cell of these two enzymes is not influenced by removal of bicarbonate. In summary, before the point of no return, morphological plasticity is associated with a corresponding plasticity of two key enzyme systems thought to be directly involved in RS formation; after the point of no return, this plasticity is lost. Analyses of this sort have thus provided additional direct evidence for the biochemical nature of the bicarbonate trigger

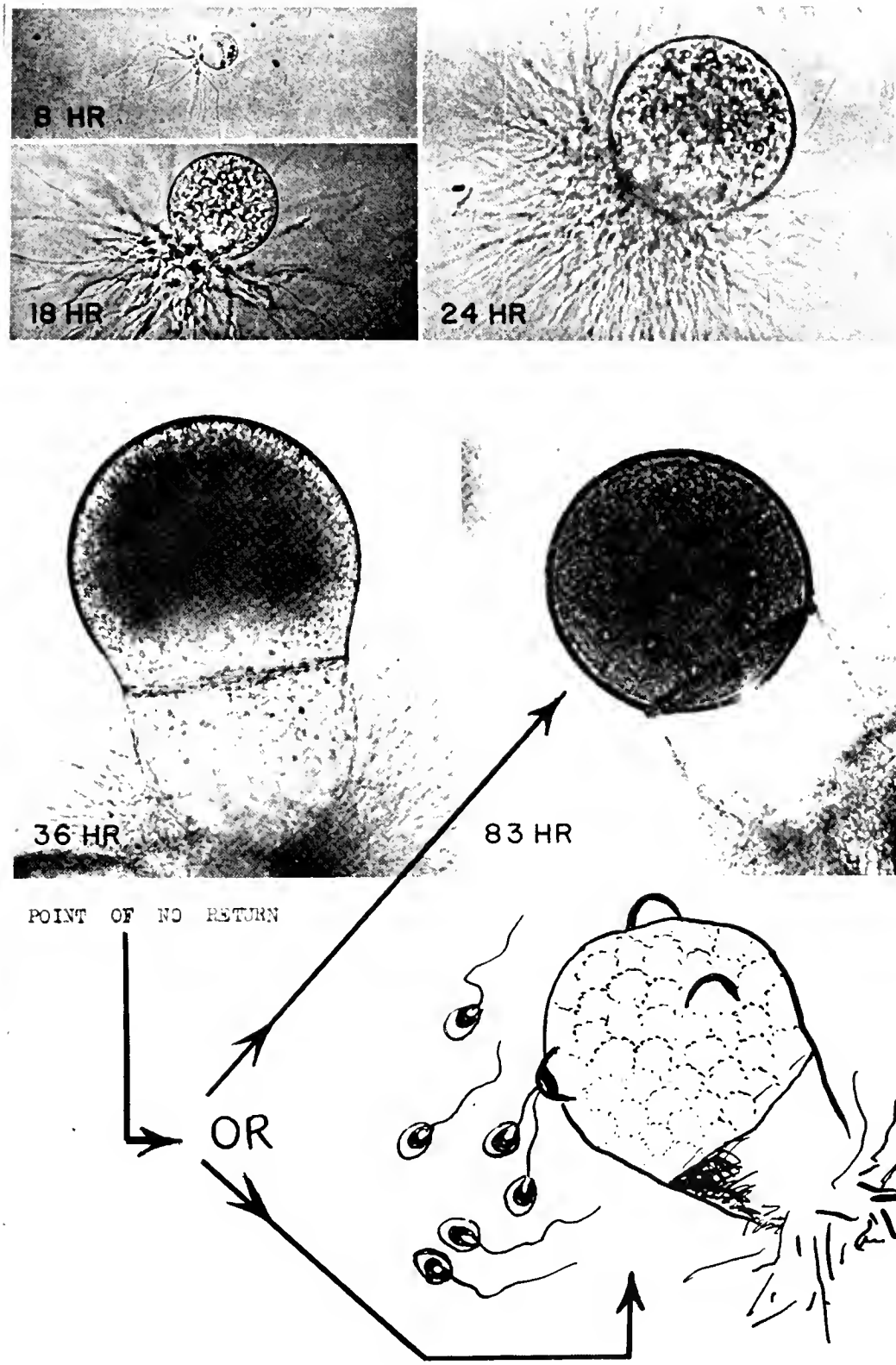


Fig. 8.

The morphoiogical point of no return during RS deveiopment in *Blastocladiella emersonii*.

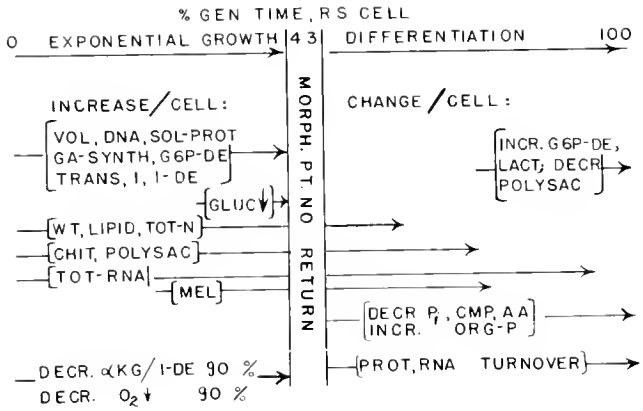


Fig. 9.

A digest of some of the events which have been quantified during exponential growth and differentiation of an RS cell.

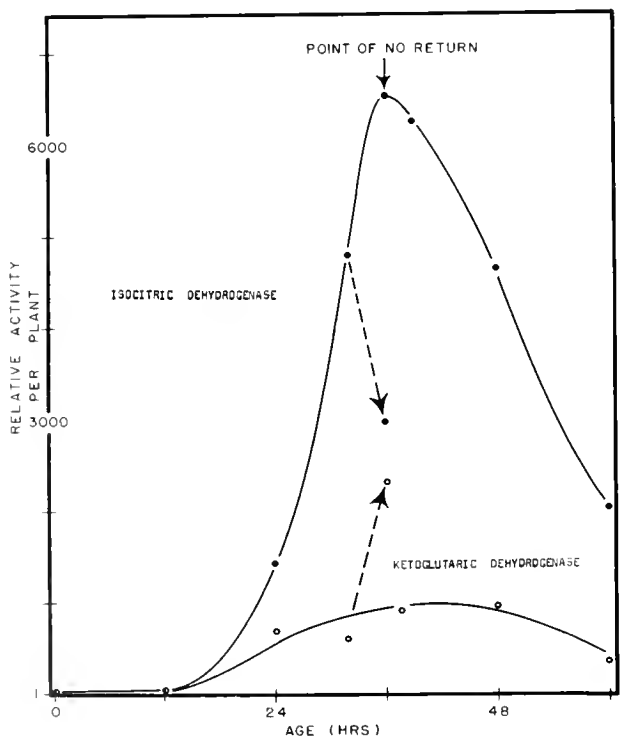


Fig. 10.

Enzymatic reversals associated with morphological reversals in *Blastocladiella emersonii*. (Fig. 1, Lovett and Cantino, *J. Gen. Microbiol. 24*, 1961; reproduced with permission of Cambridge University Press.)

mechanism. This system is currently being exploited further, and in greater depth.

Recently, we have also turned some of our attention to further exploration of the source and intracellular localization of the reducing power necessary for driving the reductive carboxylation of ketoglutarate to isocitrate. Some ten years ago, Dr. Horenstein and I found that RS cells of *B. emersonii* possessed a polyphenol oxidase system which, in crude cell-free preparations, mediated electron transfer from tyrosine to either oxygen or TPN (but not DPN). As was to be expected, this system could be coupled *in vitro* with isocitric dehydrogenase to drive reductive carboxylation of ketoglutarate to isocitrate (Fig. 11). This tyrosinase, which is not formed by the OC cell, thus constitutes one source of reducing power for the bicarbonate trigger mechanism in RS morphogenesis. Unfortunately, the enzyme is firmly bound to the RS wall and difficult to solubilize; thus, little more has been done with it so far.

A second source of reduced TPN in *Blastocladiella* is glucose-6-phosphate dehydrogenase (G6PDH). However, unlike the tyrosinase, which

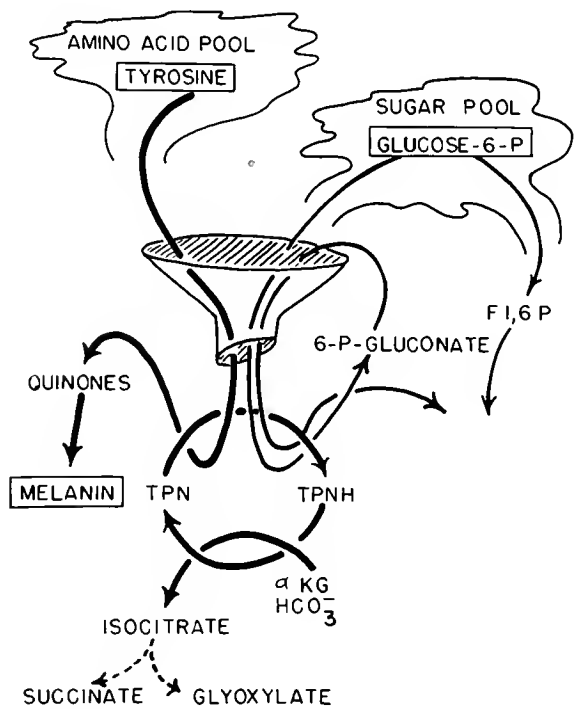


Fig. 11.

The two metabolic processes presumably involved in the generation of reducing power for carboxylation of α-ketoglutarate.

is induced to form *de novo* by bicarbonate, the G6PDH is present in both OC and RS cells. We have had some reasons to suspect, however, that bicarbonate induction of morphogenesis is associated with a bicarbonate-induced compartmentation of G6PDH within the cell. We have set up the hypothesis (Fig. 12) that (a) during the development of an OC cell, intracellular G6PDH is soluble, but that (b) during the exponential development of an RS cell, bicarbonate induces differential distribution and/or differential synthesis of this enzyme in such a way that it becomes localized on or near the cell wall or the membranes associated with it, and that (c) after the point of no return in RS development, soluble enzyme once again appears inside the cell (either via release of wall-bound enzyme into the soluble pool, or destruction of the wall-bound enzyme and concomitant *de novo* synthesis of soluble G6PDH, or some combination of these two). Some of the evidence follows.

To begin with, if the notion has validity, one might expect that during RS development the exponential rate of synthesis of total intracellular G6PDH would reflect (or at least be more nearly similar to) the exponential rate of deposition of the surface area of the cell rather than its weight or volume. Conversely, for the OC cell, one might expect the opposite to hold true. The data available suggest that this is, indeed, the case (Fig. 13). Thus, the *in vivo* evidence, although it does not prove the point, is consistent with the notion expressed in Fig. 12.

With these results sufficiently suggestive, Dr. Prem Pandhi and I have begun *in vitro* studies of *Blastocladiella's* G6PDH. Although attempts to purify it by conventional means (fractionations with ammonium sulfate, acetone, DEAE-cellulose, etc.) have only led, thus far, to several-fold increases in specific activity, experiments designed to test the hypothesis in Fig. 12 are yielding evidence in its favor. For example, when 36 hour RS cells are homogenized in 0.005 *M* TRIS-HCl buffer containing 0.001 *M* EDTA and then centrifuged at 112,000 x G, about 98% of all the G6PDH activity is in a soluble form (HSS in Fig. 14). When the pellet is extracted three times in succession with 0.005 *M* TRIS-HCl buffer, the remaining 1-2% of the G6PDH activity comes out - most of it in the first wash (1W in Fig. 14); this is the amount one would expect to find if it had simply been trapped in the fluid volume held back by the pellet. Only traces of activity are found in the second and third washes (2W and 3W in Fig. 14). A final extraction of the pellet with 1 *M* TRIS-HCl (1M in Fig. 14) yields insignificant activity.

However, similar analyses of RS cells undergoing exponential growth - in this case 24 hour cells - yield quite different results (Fig. 14). Only about 40% of the total G6PDH activity is directly soluble in 112,000 x G supernatants. The first wash yields about half as much again of the enzyme, a great deal more than one would expect if it had simply been trapped in the pellet. The second and third washes yield additional quantities of G6PDH activity, and the final extraction with 1.0 *M* TRIS-HCl yields another 20%. Note, too, that the specific activities of the enzyme in the washes do not vary greatly from one another (labeled "S.A." in the figure). Thus, as seen in the insert in Fig. 14, essentially all of the G6PDH in a 36 hour RS cell is soluble. But in a 24 hour RS cell which is growing exponentially and has not reached its point of no return, less than half of the G6PDH is soluble; more than half of it appears to be "bound" - albeit loosely "bound," since it behaves as if it were partitioning between two phases during successive extractions.

Dr. Pandhi and I are now in the process of tracking the soluble and insoluble G6PDH throughout the ontogeny of RS and OC cells; I would like to show you some of the things we

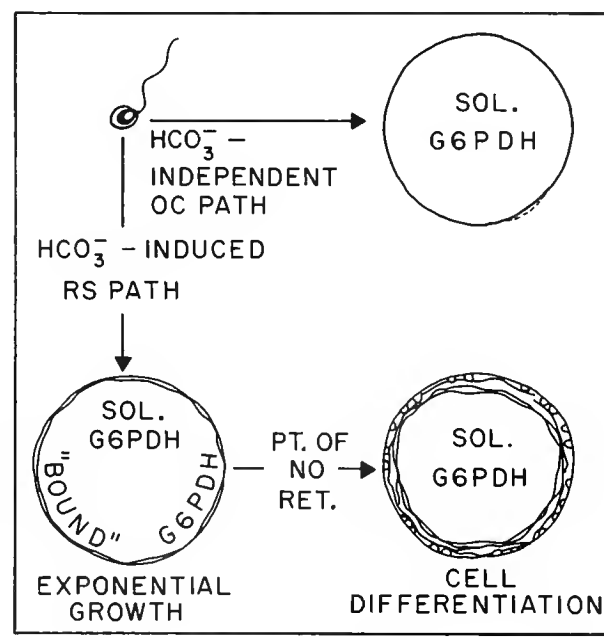


Fig. 12.

Hypothesis regarding the effect of bicarbonate on glucose-6-phosphate dehydrogenease in *Blastocladiella emersonii.*

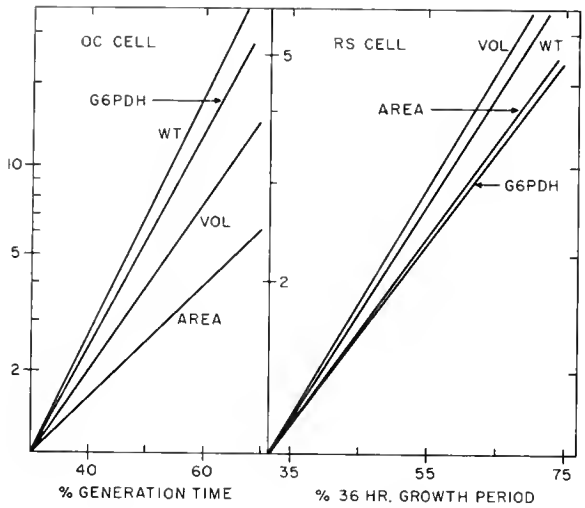


Fig. 13.

Comparative exponential rates of synthesis of glucose-6-phosphate dehydrogenase, weight, volume and area by OC and RS cells.

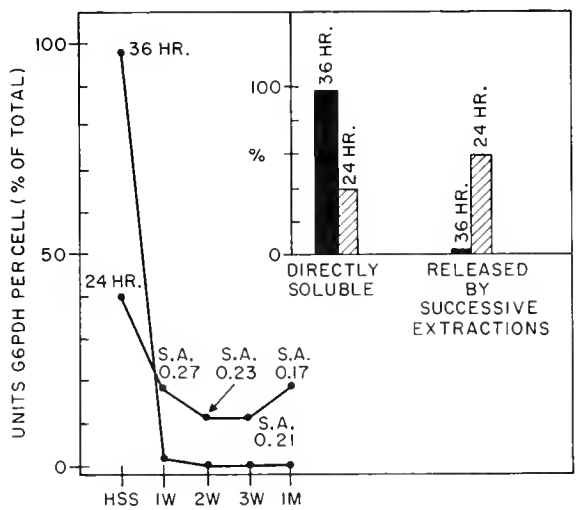


Fig. 14.

Differential solubilities of glucose-6-phosphate dehydrogenase in 24 hr and 36 hr RS cells.

have seen so far (Fig. 15). All assays were done as in the foregoing experiment. The plot in the figure shows that during the early stages of exponential growth, most of the enzyme is insoluble. But as the RS cell approaches the end of its period of exponential growth, the % soluble G6PDH gradually increases until, by the

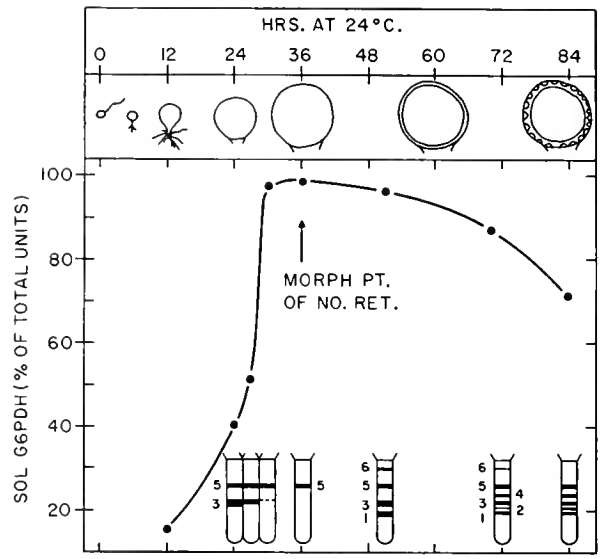


Fig. 15.

Changes in quantity and isozymic composition of the soluble G6PDH during ontogeny of an RS cell.

time the point of no return in morphogenesis is reached, essentially all of it is in soluble form. This state of affairs persists for many hours after the point of no return, but some "bound" enzyme appears again as the RS cell approaches maturity (i.e., 84 hours). So far, the data generally support the hypothesis shown in Fig. 12.

In order to obtain more definitive and informative data about the changes which occur in this enzyme during cell differentiation, we have begun to categorize its soluble and "bound" forms via disc electrophoresis in polyacrylamide gel, using a TRIS-HCl-EDTA-Borate buffer at pH 8.3. Although only a beginning has been made, the patterns obtained (Fig. 15) for the soluble enzyme reveal that striking changes in its composition occur as the RS cell moves along in its ontogeny. Two major bands (5 and 3) are present at 24 hours during exponential growth, but as growth continues band 3 gradually disappears and, by the time the point of no return is reached, only band 5 remains. After the point of no return, however, a new complex of bands of G6PDH activity makes it appearance along with band 5 which is present throughout development. It is too early to speculate as to the role of these isozymes in the bicarbonate trigger mechanism, but we have every expectation, now, that this approach will produce significant results.

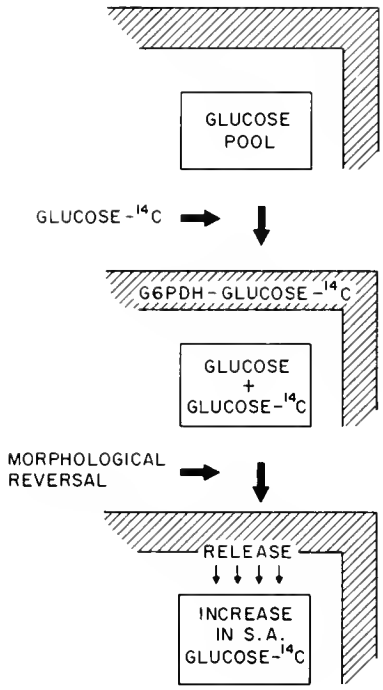


Fig. 16.

Hypothesis regarding effect of morphological reversal on the specific activity of free glucose in an RS cell.

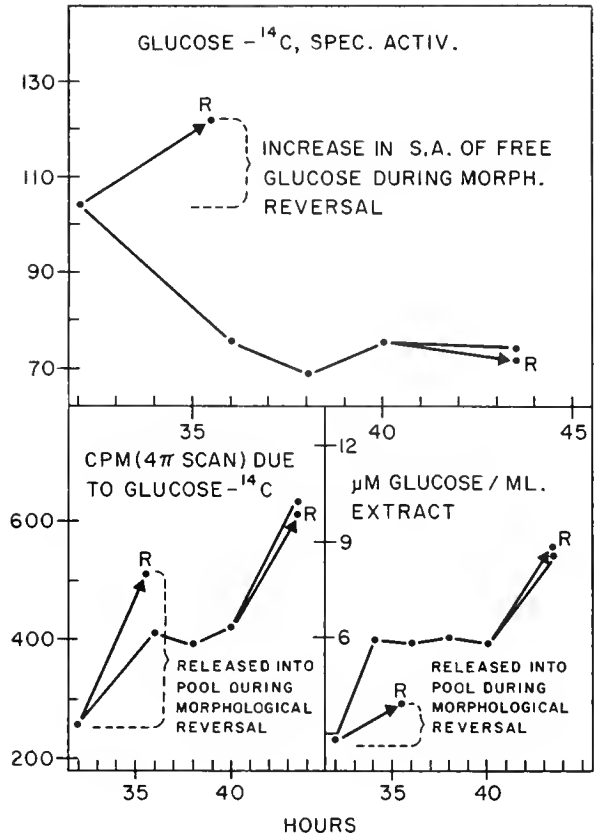


Fig. 17.

Effect of morphological reversal on the specific activity of the free pool of labeled glucose in an RS cell.

Some months ago, I also tried to approach this problem in another way, arguing as follows. If the notion outlined in Fig. 12 is valid, the RS cell could be grown in such a fashion that an unlabeled glucose pool could be created in it and detectable some hours before the end of exponential growth, i.e., before the point of no return (cf. Fig. 16). At this point, glucose-^{14}C could be fed to it, and the RS cell allowed to continue its normal growth. If G6PDH were functioning at or near the cell surface, differential localization of glucose-^{14}C and/or the immediate products of its metabolism might also occur near the cell surface. Presumably, some of this labeled glucose (or glucose derivatives) would also leak into, and mix with, the unlabeled free pool. If, now, morphological reversal were induced by removing the bicarbonate before the point of no return (where, as seen previously, a shift in isocitric and ketoglutaric dehydrogenases does indeed occur), perhaps the surface sites to which G6PDH was presumably loosely bound would also be affected, thus releasing soluble G6PDH. If this release of enzyme were also to give rise to release of the glucose (or derivatives) previously localized at these sites, specific activity of the free pool of labeled glucose in the cell would increase.

The results of such an experiment are shown in Fig. 17. Glucose-^{14}C was introduced into a synchronized culture of RS cells and the total intracellular pool (labeled plus unlabeled) of free glucose at successive stages in their ontogeny was determined with the "Glucostat" reagent. The results are shown by the continuous line in the lower right hand figure. At 32 hr. (i.e., before the point of no return) and at 40 hr. (i.e., after the point of no return) samples of these labeled cells were also transferred to water for 3 hr.; morphological reversal occurred in the former but not in the latter. The changes in the total intracellular pool of free glucose associated with the successful and unsuccessful morphological reversals are shown by the arrows in the same figure.

Samples of the glucose from each pool were then purified extensively by way of paper and

column chromatography to constant specific activity; the total counts per minute attributable to this purified glucose are shown in the lower left hand figure. The two sets of data, when combined, yielded the specific activities of the glucose pools in RS cells of different ages - including reversals - shown in the upper figure. By way of summary, morphological reversal before the point of no return does, indeed, bring about an increase in the specific activity of the glucose in the cell's free pool; after the point of no return, this does not occur. Once again, although these results could be interpreted in several ways, they are consistent with the theory we have been discussing.

Obviously, much more work is needed, and we are in the process of doing some of it. In conclusion, *Blastocladiella emersonii* provides a very satisfactory system for studying the relations between biochemical and morphological differentiation (2). I am sure that Dr. Lovett, who now follows me on this program, will illustrate in yet another way that this is so.

SCHRAER: What is the ecology of this organism?

CANTINO: It is found in fairly slow-moving bodies of water, sometimes streams but more often ponds and puddles, and in soil. The genus is ubiquitous, but for this particular species I cannot say definitely. But what do you mean by ecology, in particular?

SCHRAER: I was referring to the oxygen content of the water in which they are found.

CANTINO: It likes to grow in fairly well aerated bodies of water, but it is unhappy, apparently, in bodies of water heavily laden with organic matter. It can be trapped on insect exoskeleta, fruits of the *Rosaceae*, etc.

ZIMMERMAN: Does the CO_2 content in these waters vary sufficiently to give you the either/or type of growth?

CANTINO: I think so, but not directly because of the CO_2 content of the water. Rather, it is the fact that when this organism grows in nature, it is often surrounded by microflora and fauna which tend to localize around it. I feel certain, although I've never stuck microelectrodes therein, that the CO_2 concentration within such localized "pockets" must be higher than on the outside, and that this may be involved in the induction of RS differentiation in nature.

EPEL: Does isocitrate eliminate CO_2?

CANTINO: Isocitrate did not do so in experiments done years ago; we'd like to assume that this was because it does not get in very easily. Ketoglutarate does get in, and does the trick under certain conditions of nutrition. Presumably, it functions as a substrate for the backward reaction.

EPEL: Have you been able to use malic acid to affect CO_2 consumption?

CANTINO: Do you mean the Ochoa "malic" enzyme? We think it may be operative at some stages, but I would not care to say "yes" or "no" because these assays were done about ten years ago when we were not using synchronized cultures. Therefore, I'm not sure of the significance of these old assays.

KAHN: How do you visualize the control of enzyme levels or activity?

CANTINO: I have no satisfactory basis for speculation on this point.

CHALKLEY: Is there DNA synthesis at the point of no return?

CANTINO: Net DNA synthesis ceases at the point of no return. If we plot the DNA/cell (using older methods of extraction of some 6-7 years ago) against developmental age, the curve rises and then levels off almost precisely at the point of no return. There is no additional net synthesis of DNA after this point, although net synthesis of RNA continues. However, during the period after the point of no return, the composition (base ratios and physical properties) of the RNA begins to change. (see subsequent reply by CANTINO to a question by GROSS).

J. WRIGHT: I gather, Dr. Lovett, that you're going to discuss this in the next paper?

LOVETT: Yes, but I won't discuss that part of the life cycle.

KAHN: Is the DNA synthesis synchronous?

CANTINO: If you mean synchronous in terms of nuclear division, we don't know. For RNA, we have more data, but I would rather not go out on a limb even here.

GROSS: Are there mutants of this organism that are incapable of making the switch?

CANTINO: Yes, during the past 18 years we've isolated four spontaneous mutants during essentially day-to-day observations of cultures growing on plates. So, they occur with a low frequency. The mutants are incapable of responding to the bicarbonate trigger mechanism or, as far as we know, to any other "inducer"; they will not form RS. When some were analyzed for their enzyme contents, they were found to lack ketoglutaric dehydrogenase (and aconitase).

GROSS: Do they lack it entirely? They don't have the characteristic response?

CANTINO: No, they won't respond; they won't produce RS in response to bicarbonate. (Note added in proof: The mutants on which

these assays were done were lost some years ago. Their content of ketoglutaric dehydrogenase activity, based on assays of multiple generation cultures in 1953, was not absolutely zero, but about 4% of the level found in the wild type; see Table I).

J. WRIGHT: They won't respond to any other system you've tried?

CANTINO: That's right. They seemed to have lost the capacity. We would like to think this lesion involved some kind of a "master gene" which exerted pleomorphic effects because so many, many things (including extreme reduction in viability) were associated with this loss of capacity for the formation of an RS cell.

GRUN: In your discussion, you've been dealing with RS as a unit, as something fairly constant, but in the beginning slides you were stressing RS as being highly variant, oranges ones, light ones, etc. How do you account for the variability in RS?

CANTINO: Well, I didn't stress the variability of RS at the beginning. I stressed that if one starts with a population of spores, they have the capacity to develop along at least four alternate pathways, although I may not have said it in so many (or rather so few) words. Spores can develop into RS cells, OC cells, orange cells, or a type which we call "late-colorless" cells. Thus, there are four alternate pathways in the life history of *B. emersonii* but the ones I have discussed today are the two major ones.

GRUN: Then was this just one of the changes that occurs in this system?

CANTINO: Let me clarify. (The following was altered slightly in the proof in order to clarify the clarification which involved extensive use of the blackboard.) Starting with a population of spores on plates, one obtains two main cell types in the first generation; either OC cells or RS cells, depending upon whether or not bicarbonate is present (cf. Fig. 18). Between 99 and 100% of the population of spores will do this. However, depending upon the growth medium selected, up to 0.5% of the population of first-generation plants will consist of what we have called an "O" cell - literally, an orange cell. The cell is orange because, judging from evidence obtained with mutant strains, it contains gamma-carotene. Another zero to 0.5% of the population consists of what we have called "late-colorless" cells, cells which differ from OC cells by their much longer generation time. Does this clarify it? I have been speaking of a population of spores, not a single spore.

GRUN: Then the orange and the late-colorless are not triggered by bicarbonate, but by something else?

CANTINO: It gets fuzzier now! If we start with an orange cell, harvest all its spores, and plate them out, the new generation of plants has *essentially* the same composition obtained with the usual population of spores. However, it is not exactly the same; more nearly 1% of the population now consists of orange cells. From the spores of a late colorless cell, an essentially normal population is also obtained; but, in this case, fewer than average numbers of orange cells are produced. (The reply above was altered slightly in the proofs for purposes of clarification; the reader is referred to the paper by Cantino and Hyatt cited in the bibliography of this report for detailed tabulations of the kinds of progeny produced by the different cell types of *B. emersonii.)*

J. WRIGHT: Are these orange or late colorless cells on the periphery of a culture or are they different in some way?

CANTINO: No, when starting with spores which have been spread out uniformly on the surface of a Petri dish so that each one develops into an individual plant, you find that these various cell types are distributed essentially at random. We have published some evidence to show that the distribution of a cytoplasmic particle, which we labeled a "gamma" particle, may be involved (3).

McCARL: I have a question on the glucose-6-phosphate dehydrogenase. Do you feel that it's synthesized on the surface of the membrane?

TABLE I

Enzyme system assayed* (crude cell-free preparations)	Specific Activity in	
	Wild-type OC cells	Mutant cells
Cytochrome $C_{(RED)}$ oxidation	111	114
Succinic dehydrogenase	125	126
Isocitric dehydrogenase	160	156
Malic dehydrogenase	220	74
Fumarase	148	70
Aconitase	108	0
Ketoglutaric dehydrogenase	94	4

*For details see reference 5.

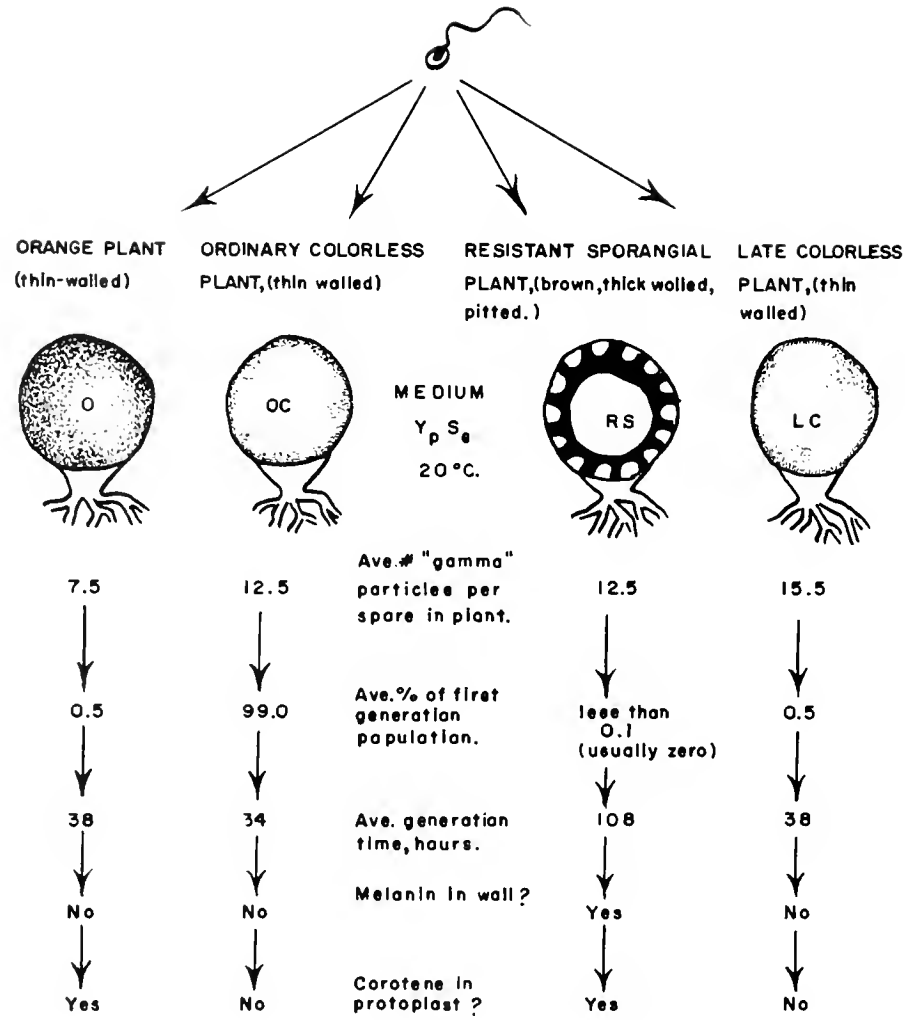


Fig. 18.

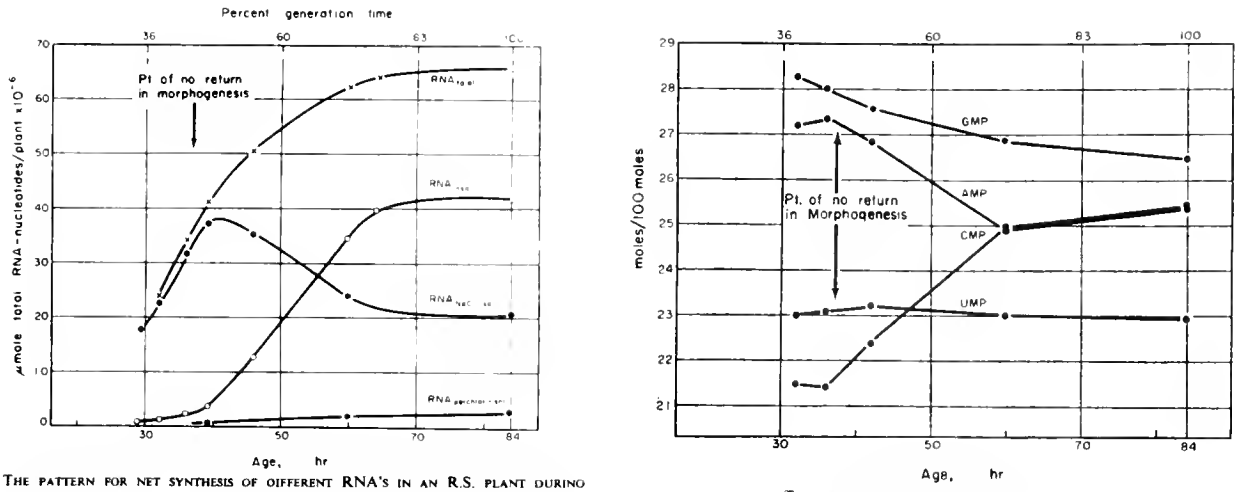
The four alternate pathways of development in *Blastocladiella emersonii*. (Fig. 2, Cantino, *In* "11th Symp. of the Soc. for Gen. Microbiol.," 1961; reproduced with permission of the Society for General Microbiology.)

CANTINO: Well, this will be pure guesswork; my working hypothesis is that it is synthesized on ribosomal particles close to the membranes near the wall.

FERGUS: To go back to a former question, if you sample a number of individual sporangia, would you still get this ratio of the four types within a single sporangium?

CANTINO: Yes, provided the environment is favorable. One can alter these ratios quite easily by modification of the media. For example, 60% of the spores in a population can be induced to produce orange cells if a suitable concentration of actidione is added (note added in proof: - and if peptone, which tends to repress genesis of orange cells, is not present). On the other hand, addition of diphenylamine eliminates orange cells from a population.

GROSS: These data that you've been discussing about the population that can result from germinating spores suggest to me that you're thinking about the mechanism of this differentiative process, probably, the way Barbara Wright was thinking about hers. That is this (and tell me if I'm doing either of you an injustice): I have the feeling you are thinking of this as a modulation process. The enzymatic systems that control the product, giving a physically different kind of cell, are all there. What's really critical is that the levels of interaction



THE PATTERN FOR NET SYNTHESIS OF DIFFERENT RNA'S IN AN R.S. PLANT DURING DIFFERENTIATION.
Values for RNA are expressed as total μMoles of all nucleotides derived from the RNA by KOH hydrolysis.

THE MOLAR COMPOSITION OF $RNA_{NaCl\text{-}sol}$ Isolated at different stages during differentiation of an R.S. plant of *B. emersonii*.

Fig. 19.

Transformations in the extractability and composition of the RNA of *Blastocladiella emersonii* during RS differentiation. (Figs. 2, 3, Cantino, *Phytochemistry 1*, 1961; reproduced with permission of Pergamon Press Limited.)

change in some way so that the system is modulated and the products are different. Now, it seems to me that in both of these there is a critical way to determine whether that is the whole story. That is to find out if at some point before the point of no return you can disable the genome. You disable the genes so that no information can flow from them. If in that instance this process continues, it's clear that this is a modulation. It's differentiation, to be sure, but without benefit of gene action. Now, on the other hand, if, in the condition where we stop the gene action, this too stops, then you know you've got to have something additional to the simple modulation of the rates of enzymatic processes and the way that they interact.

CANTINO: I'm inclined to agree (but I have no direct evidence which will bear on this point). However, may I summarize some analytical results obtained in 1960 which may have an *indirect* bearing here? (The following reply is an expanded version of the original one; the data can be found in ref. 4). Essentially all of the RNA in a growing RS cell is soluble in hot NaCl (= $RNA_{NaCl\text{-}sol}$), and it's molar composition (CMP:AMP:UMP:GMP = 1.00:1.33:1.08:1.27) stays constant during the last stages of exponential growth before the point of no return is reached. But, after the point of no return, the quantity of $RNA_{NaCl\text{-}sol}$ per cell begins to decrease again, and simultaneously it undergoes a sharp change in composition (Fig. 19). Just before the amount per cell of this NaCl-soluble RNA reaches its peak, a new RNA appears in the cell. This new RNA is attached to cell particles which sediment at 10,000 to 15,000 x G, it is insoluble in hot NaCl but detectable by KOH hydrolysis to yield its component nucleotides, and these nucleotides are present in it in almost exactly equal quantities (CMP:AMP:UMP:GMP = 1.00:1.00:1.00:1.03). This insoluble RNA (RNA_{insol}), as seen in the figure, rises sharply in the cell immediately after the point of no return, and it reaches its maximum level when the cell is about 70 hr. old. The plot for RNA_{total} in the figure was simply gotten by adding up the data for RNA_{insol} and $RNA_{NaCl\text{-}sol}$. Assays for these two RNA types were also made in RS cells which had been induced to undergo morphological reversal. The data (not shown in this figure) revealed that just before the point of no return, when RNA_{insol} first becomes detectable, morphogenetic reversal induced a sudden loss of about half of this RNA_{insol}. Similar experiments performed after the point of no return did not induce the shift.

DEERING: I have something that might be relevant to this. I've started some work on the effects of ultraviolet light on the OC development of this organism. Ultraviolet light is be-

lieved to act mainly on the nucleic acids, RNA and DNA in many biological systems. In our experiments we've watched the morphological development of the plants after UV irradiation of the spores. Almost independent of the dose of ultraviolet light, these plants will go through the early stages of development to a point approximately 60% of the way through the normal life cycle and then they'll essentially stop. The development up to that point seems to be quite normal except that it proceeds more slowly, the higher the UV dose. We're currently starting studies of nuclear counts, and RNA and DNA content, after exposure of the spores to ultraviolet light. In the OC form, the point 60% of the way through the life cycle, at which growth stops after UV, corresponds to some of the other changes that Cantino has just talked about. This suggests that some critical changes, blocked by earlier UV irradiation, are going on at this point in the life cycle. This point is approximately the point of no return from OC to RS. By that I mean there is a point in the OC development beyond which you can't add bicarbonate and make it go to the RS form.

CANTINO: That's right, it is about the point of no return for OC cells. I want to add that this applies only to synchronized plate cultures of OC cells. The point of no return for OC cells grown in liquid cultures is another matter.

DEERING: These experiments were on synchronized plate cultures. After the UV, the development of these plants stops roughly at the "point of no return." This might indicate that some damage to the RNA or DNA (or both) in the spores has stopped them from supplying information necessary to get them beyond a certain point in development. This might indirectly implicate the involvement of nucleic acids in this change.

CANTINO: There is one thing I didn't mention in respect to the data for RNA shown in Fig. 19. First of all, because of the sharp changes in base ratios and in the quantities of RNA_{insol} and $RNA_{NaCl\text{-}sol}$, the conclusion seems inescapable that a large amount of RNA turnover is occurring after the point of no return in an RS cell. (If comparable studies had been done with OC cells, the results might bear directly upon Dr. Deering's comments). Secondly, at the point of no return there occurs a sudden and very fast rise in the free CMP acid pool of the cell, but this change is not counterbalanced by a comparable rise in the pools of AMP or UMP. The CMP is reutilized, however, as the RS cell proceeds beyond its point of no return.

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NUCLEIC ACID SYNTHESIS DURING DIFFERENTIATION OF *BLASTOCLADIELLA EMERSONII*

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Because Dr. Cantino has already presented an excellent summary of the life cycle of *B. emersonii* (1), I can jump right into the material I want to discuss without further introduction. This will deal with the formation of zoospores, which he skipped over very lightly, and their germination, two phases in the life cycle that are very close together. Before starting, however, I would like to mention that most of the work on spore differentiation was done by a student in my laboratory, Sister Mary Nadine Murphy, now at Mundelein College, and the electron micrographs I will show were prepared in cooperation with Dr. A. E. Vatter, at the University of Colorado Medical School, who introduced me to the mysteries of electron microscopy.

We are interested in the formation of spores and their germination for two reasons: one general and one specific. In general, we feel that the continuous transition from a relatively undifferentiated plant with many nuclei to a large number of highly differentiated spores and the germination of these spores to give back tiny, but nevertheless very similar, plants should provide an excellent opportunity to study, and perhaps even discover, some control mechanisms for the cellular regulation of differentiation. Our specific reason is an interest in nuclei acids and, particularly, the spore nuclear cap, which is shown in the first figure (Fig. 1).*

The zoospore is characterized by the large "nuclear cap," a structure which has been known for a long time. The spores in this photograph were fixed and stained with the basic dye, toluidine blue. In addition to the barely visible flagellum, one can see the nucleus and a nucleolus. All of these structures can be seen better in Fig. 2, which is an electron micrograph

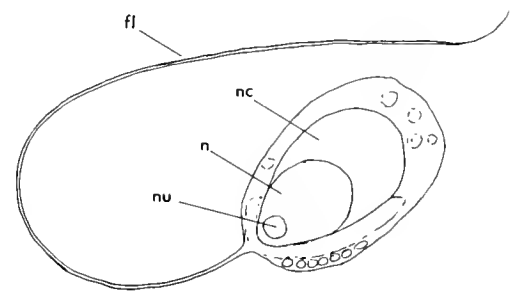

Fig. 1.

Photomicrograph of a *Blastocladiella* zoospore. nc, nuclear cap; n, nucleus; nu, nucleolus; fl, flagellum.

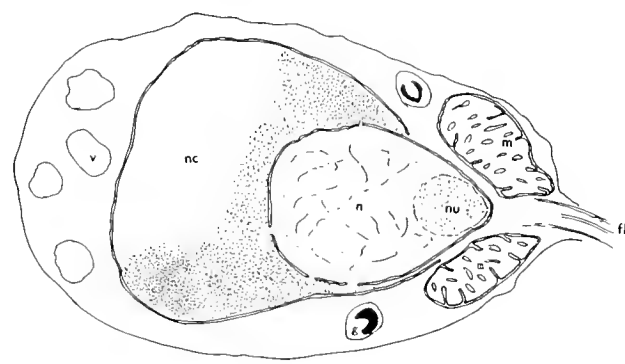

Fig. 2.

Electron micrograph of a section through a zoospore. m, mitochondrion; v, vesicle; g, unidentified granule.

*Most of the figures presented here are schematic and approximate to illustrate the material discussed. The complete curves, micrographs, and experimental details will be published elsewhere.

section through a spore. The micrograph clearly shows the large basophilic cap with its finely granular contents; note also that there is no material of a similar nature outside the cap. It also shows the nucleus, the nucleolus, and, although it looks like two, the single large mitochondrion with the flagellum coming out through the bottom.

Figure 3 is a tangential section through the nucleus showing partial sections of the nucleus and the cap with the small osmiophilic particles in it. I'm sure you've guessed that these are ribosomes. That is what we think they are. The pores in the nuclear membrane are particularly obvious. If you look carefully at the membrane of the cap itself, on the other hand, it is seen to be continuous. There is a single large mitochondrion per spore; it is always acentrically located and specifically associated with lipid granules and what we suspect may turn out to be polysaccharide granules. Figure 4 is a section through the lower end of the spore showing the centriole, or basal body, at the base of the flagellum, going through the mitochondrion in a small channel. Figure 5 is a section taken at right angles to the last and shows quite clearly that the flagellum doesn't just pass by the mitochondrion; it goes right through a channel in the middle. Note the direction of the flagellar shaft with respect to the mitochondrion and the "rootlets" that extend down through other channels into the body of the mitochondrion itself. We don't have much of an idea concerning the function of these at present. The purpose of this hasty survey of zoospore morphology has been to emphasize the highly organized state of the spores; the nearest approximation I can think of would be a protozoan or perhaps a sperm cell. It is obvious that a considerable intracellular transformation must occur in the process of forming these spores at the end of the life cycle.

Several years ago, Dr. Gilbert Turian, in Switzerland, demonstrated the presence of these small particles in electron micrographs of *Allomyces* nuclear caps (2), but *Allomyces* is a filamentous organism and is, therefore, difficult to grow in synchronized cultures. We have been interested in looking at the caps in *B. emersonii*, partly because of familiarity with the organism, but partly because, in terms of differentiation, we can get much better synchrony for our work.

Figure 6 shows that one can readily isolate the nuclear caps from zoospores. Because the caps were central to our interests, we wanted to be sure we could get them out, characterize the contents, and be certain they were what we thought they were, that is to say, ribosomes. By proper procedures, one can gently rupture the spores, separate the caps, and purify them by differential centrifugation (3). When we did this, we found that they were composed of about 40% RNA, 60% protein, and made up about 70% of the total RNA of the cell. They also contributed something like 18% of the total dry weight of the spore. This was all consistent with a ribosomal composition.

Analysis of the purified caps demonstrated that they matched ribosomes in their chemical composition. When we purified the caps and then isolated their particulate contents, we could also show that they were ribosome-like by examining them in the analytical ultracentrifuge to obtain their sedimentation coefficient. They sediment at about 80S, a value similar to that found for other fungi. The particles also dissociate in low magnesium ion concentration and contain a latent ribonuclease. Thus, they appear to have the characteristics of ribosomes as determined by a variety of procedures. They also seem to be very pure. When the particles are isolated from caps, one obtains a glassy pellet so transparent

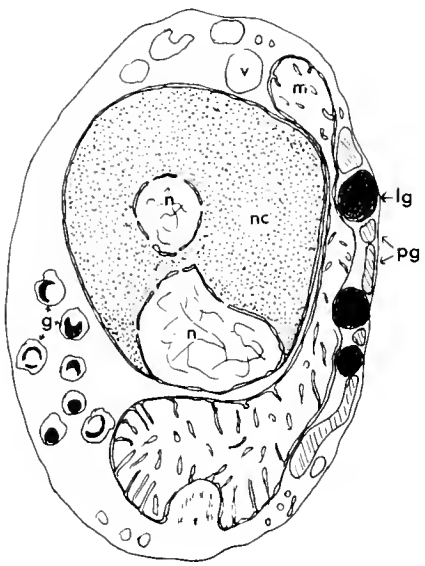

Fig. 3.

Electron micrograph of a section through a zoospore tangential to the nucleus. lg, lipid granule; pg, polysaccharide; g, granule.

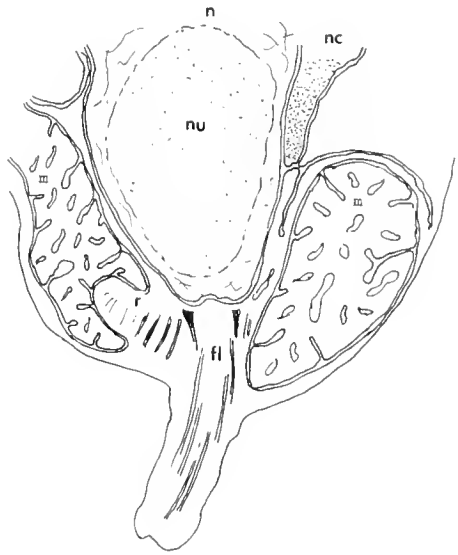

Fig. 4.

Electron micrograph of flagellar base and rootlet. r, rootlet; fl, flagellar fibrils; m, mitochondrion.

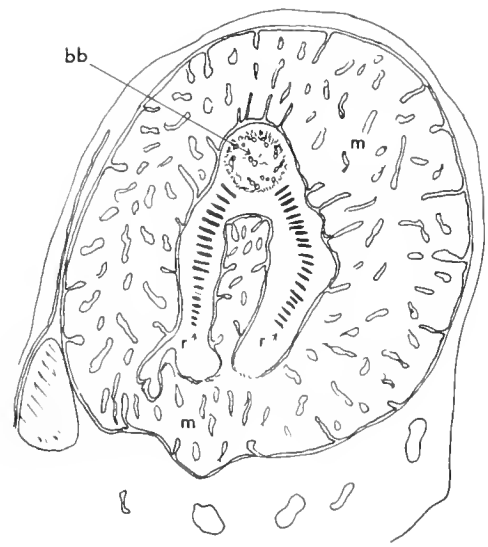

Fig. 5.

Cross section through the mitochondrion and basal body of a zoospore. bb, basal body; r, rootlet.

as to be scarcely visible in the bottom of the Spinco tube. The washed cap ribosomes contain about 63% RNA and 27% protein.

Before launching into some of the experimental work on the formation of the spores, I would like to discuss very briefly some of our ideas concerning the structure and function of the nuclear cap. The observation that all the cellular ribosomes are packaged in this peculiar structure surrounded by a membrane raised some rather obvious questions as to its function. First, where do the cap ribosomes come from? One can guess, and I think our original guess, that they come from the cytoplasm, turned out to be correct, although it obviously had to be proved. It is reasonable to expect that the spores might conserve their ribosomes. However, they might also be made essentially *in situ*, at the time the cap is formed, by degradation of pre-existing ribosomes followed by resynthesis in a new location.

A second, and perhaps even more interesting, problem concerns the function of the cap for the spore. *Blastocladiella* is not the only fungus to produce these; they are produced by a whole series of fungi. But why on earth do they form such unusual structures? First, it may be that the cap serves as a storage

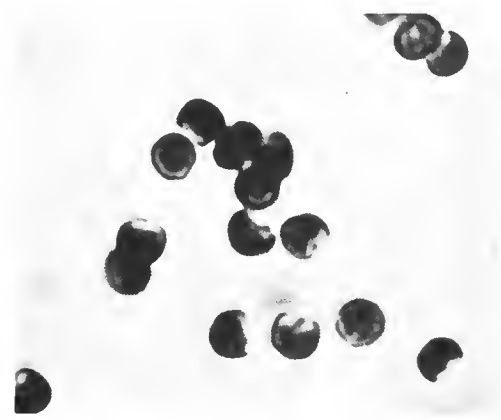

Fig. 6.

Isolated zoospore nuclear caps.

reservoir of RNA and protein for early germination. It is possible that the cell degrades the cap ribonucleoprotein and uses the products to make new ribosomes to start growth. Alternatively, it might store the ribosomes during the non-synthetic zoospore stage. I should emphasize that the zoospore is motile and metabolically active, but it doesn't grow. The

formation of a cap could be a way of protecting the ribosomes from degradation during a time when no synthesis is actually occurring. It also could be an unusual mechanism for controlling protein synthesis by isolating the ribosomes from any one of the many factors involved in the complete functional synthetic system, such as ATP from the mitochondria, for example. It could equally well serve some combination of these. I am stressing these points because I think they provide us with some ideas that can be tested.

For example, if the cap merely conserves the ribonucleoprotein for synthesis of new ribosomes, germination, then, it ought to be associated with or require concomitant ribosome synthesis at an early stage. If, on the other hand, the ribosomes are conserved as functional units and are actually used as ribosomes without alteration, then germination and early protein synthesis might be quite independent of ribosomal RNA synthesis but could require "messenger" RNA synthesis or coding of some kind. Alternatively, if the ribosomes are functional, the spore could already be precoded and ready to go; in fact the ribosomes could have the information stored away with them in such a manner that only release from the cap would be necessary. In this case, germination might be completely independent of early RNA synthesis of any kind.

I think we can test these hypotheses. We can isolate the caps and look at them in a cell-free system, for example, to estimate their functional capacity *in vitro*. We have been trying to get a reliable cell-free system to do this, but we have not yet been successful.

I would now like to talk about differentiation in terms of the source of the cap ribosomes and then briefly discuss the process of germination, which presents additional clues concerning the particular problem of cap function.

CHALKLEY: Have you looked at these under the electron microscope to see if there are polysomes there?

LOVETT: We haven't looked at them in the electron microscope, but we have tried a few inconclusive experiments by isolating the caps, lysing them very gently with detergents, and then layering them on gradients to look for polysomes. This should show us if there are lots of them. So far, we don't find any. However, we haven't done enough of this to be sure.

I want to turn to the formation of zoospores, and RNA synthesis in particular, although I'll mention a few other things. First, what happens during the differentiation to form zoospores? Figure 7 is a summary diagrm illustrating a part of the life cycle starting with the tiny spore and extending through the exponential growth phase to the mature plant containing many nuclei. Under our conditions, the number of nuclei turns out to be very close to 256, which is somewhat different from plants grown by Dr. Cantino's method. Formation of the papillae and subsequent events lead to the formation of the zoospores. This graph is a plot of per cent papilla formation versus culture age to show how sharply the transition occurs in our system. We grow the cultures by inoculating zoospores into a rich medium and then aerating and stirring at 24°C (4). At 15½ hr. we induce differentiation by changing the medium; we don't wait for it to occur normally, although it will do so without induction. However, we get much better synchrony by changing the medium to induce differentiation. This is represented by the fact that the entire population enters this papilla stage, the first obvious morphological event, within a span of 1 hr, and 60% of the plants form papillae within a 24 min interval. This is pretty good synchrony for an organism with a life cycle of approximately 24 hr.

DEERING: How do you induce this?

LOVETT: We just change the medium. We wash out the growth medium and resuspend the plants in a dilute inorganic salt solution, the 1/2DS you will see indicated in some of the figures. Figure 8 summarizes some of the data obtained for the total protein, RNA and DNA of cultures. You can see that until 15½ hr, when the medium was changed, all these increased exponentially, but shortly after the change they began to level off: DNA and RNA at about 16½ hr and protein and dry weight at about 17 or 17½ hr.

Figure 9 illustrates a little more dramatically, in a nonlogarithmic plot, the pattern observed for whole-cell RNA. You will notice that total RNA continues to increase for a while after the inducation, but then begins to go downhill, and does so continuously until the end of the cycle at 19½ hr when the spores are discharged. This finally represents a 35% loss in the total RNA of the plant.

We were interested in these RNA changes, and Figure 10 shows that not only does RNA start to disappear after 16½ hr, but, if one measures short-term pulse-labeling with C^{14}-uracil, the rate of incorporation also drops very drastically between 16 and 17 hr. I want to emphasize the morphological point: the heavy

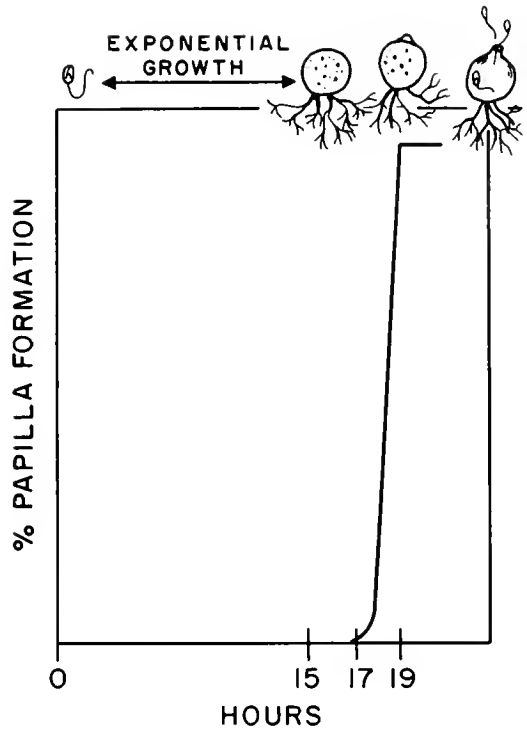

Fig. 7.

Papilla formation as a function of culture age.

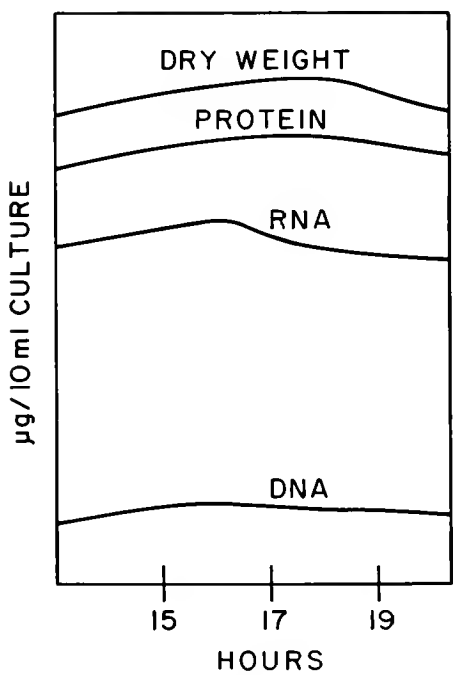

Fig. 8.

Semi-log plot of major cell constituents.

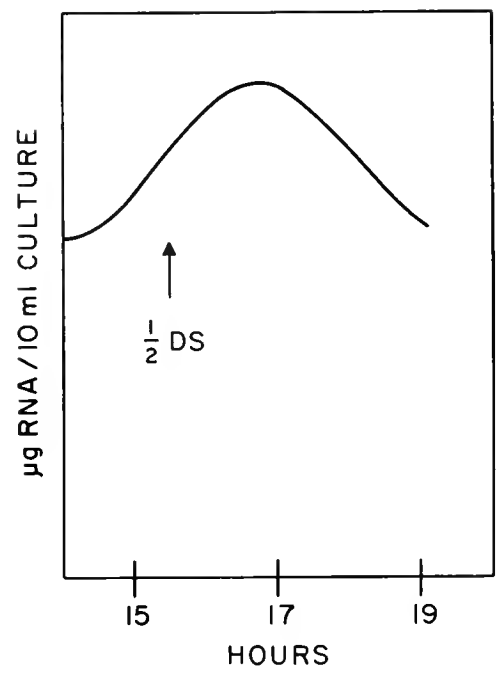

Fig. 9.

Total RNA content vs time.

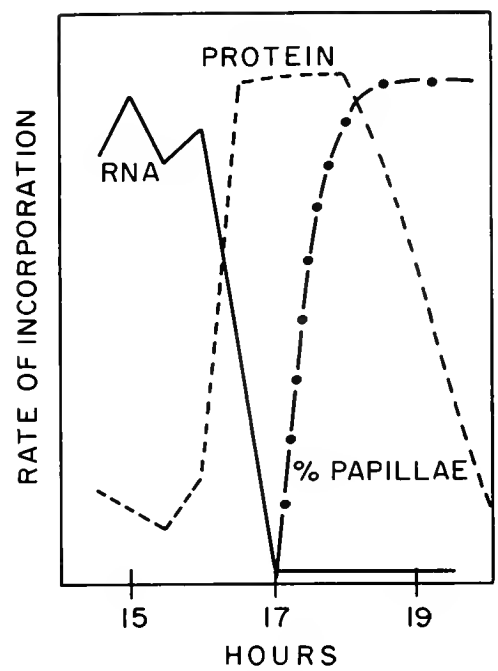

Fig. 10.

Incorporation of RNA and protein precursors.

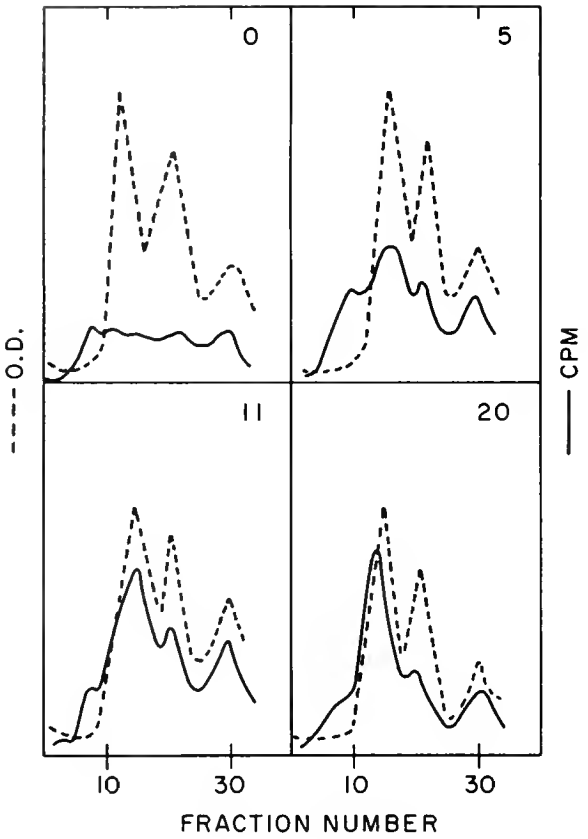

Fig. 11.

Sedimentation profiles of RNA in a pulse-chase experiment at 16 hr.

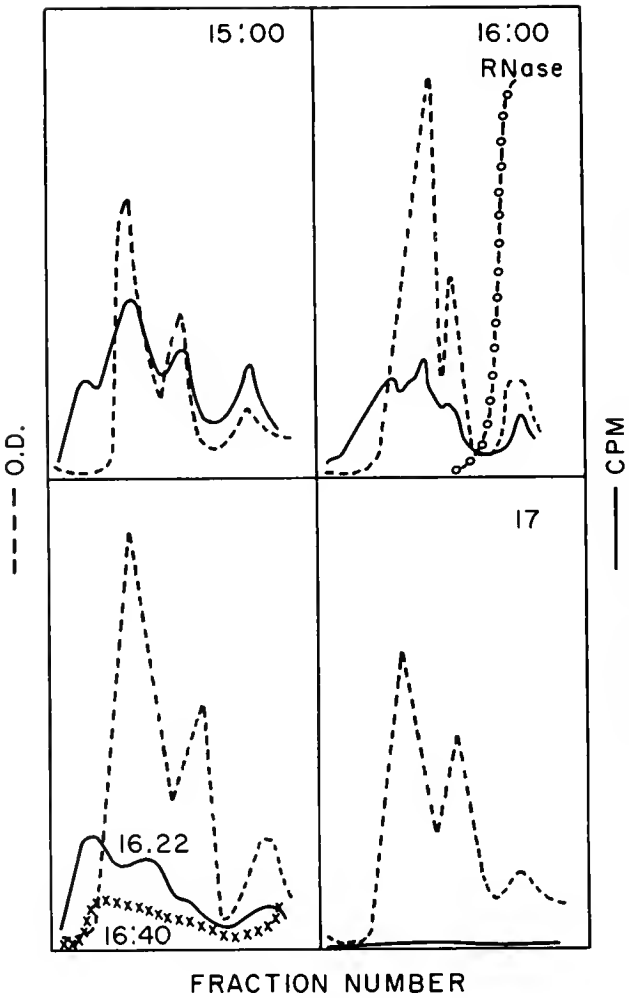

Fig. 12.

Sedimentation profiles of pulse-labeled RNA prepared at different stages of development.

dashed curve in this figure indicates the pattern of papilla formation in the same culture. It was followed in all cultures, but is not always plotted in the figures. It demonstrates that we have a good, synchronized culture and that RNA synthesis essentially stops before the appearance of the first morphological event. I should perhaps say that gross RNA synthesis has stopped. The same figure illustrates the pattern of leucine incorporation. You will notice that this goes up and continues to rise when RNA synthesis is dropping. It continues longer and drops more slowly, although it has reached a low level by 19½ hr.

DEERING: Is this label in the dilute salt solution that you put in after you wash these?

LOVETT: Every experiment I'm talking about now is in the dilute salt solution after 15½ hr.

DEERING: Then the label is added after 15½ hr?

LOVETT: Well, we often check points before. In this experiment a few points were taken before 15½ hr, which is the reason for the drop and subsequent rise in the RNA incorporation curve. Changing the medium does modify this somewhat. We don't, however, attach much significance to this at present.

The fact that uracil incorporation seemed to stop so drastically and sharply suggested that the cells were obviously turning off RNA synthesis and, perhaps, undergoing considerable degradation and turnover. As I said earlier, we felt it important to determine where the nuclear

cap ribosomes came from. If a great deal of turnover was going on, it could mean that the ribosomes were actually being degraded and resynthesized and, in this way, being redistributed from one part of the cell to another.

The results of a pulse-chase experiment at 16 hr are given in Fig. 11 and show that we could do labeling and density gradient experiments to study RNA synthesis. For this experiment the plants were pulsed with C^{14}-uracil for 2 min and then "chased" with an excess of "cold" uracil. Zero min indicates the RNA at the start of the chase. The other graphs show the pattern of labeling during the "chase." The first surprising thing we noticed was that these so-called plants have a very animal-like characteristic; they produce a rapidly labeled, heavy RNA that we think, on somewhat indirect evidence, is actually a precursor of ribosomes. It labels rapidly and disappears during the chase as the radioactivity increases under the ribosomal peaks. Actually, we suggest this on the basis of comparison with work on animal tissues where other people have drawn similar conclusions (5). We haven't, however, identified it or proven it to be a ribosomal precursor. To do this it will be necessary to isolate it and do much more work with it. This experiment was done at 16 hr, just before all the interesting events occur.

Figure 12 illustrates the pattern of RNA synthesis as a function of time. Sixteen hr, 40 min is just before synthesis is apparently shut down. The experiment is similar to that on the last slide, except that in this case we only exposed the plants to C^{14}-uracil for 10 min, then killed them and extracted the RNA for gradient centrifugation. I only want to point out that this is a typical pattern for transfer-, ribosomal-, and heavy-RNA incorporation, but that these steadily decrease starting at 16 hr until, by 17 hr, you can't detect any label whatsoever in the RNA fractions. The heavy RNA peak seems to be the last to disappear.

TS'O: What is the ribonuclease treatment?

LOVETT: The curve for ribonuclease-treated RNA just shows that if you treat the RNA's in any of these stages, of which we've only shown one, with ribonuclease, that all your material ends up at the top of the gradient. You don't have any in the region of the O.D. peaks, as in the untreated samples.

This seemed nicely consistent with our earlier results on total RNA and post-labeling, etc., but when we started looking at the pools in terms of Barbara Wright's work (6), which

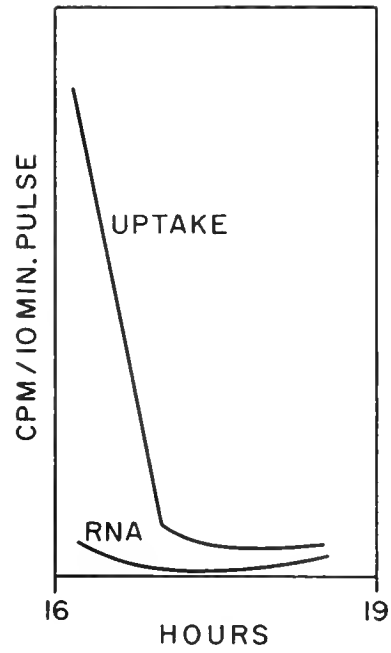

Fig. 13.

Whole cell uptake vs incorporation into RNA.

we needed to do, we suddenly discovered that total uptake versus the amount incorporated into RNA changed drastically during the same period. In other words, there was about a 13-fold decrease in the ability of uracil to enter the pools between slightly after 16 hr and 17 hr. This meant that we had to re-evaluate our previous interpretation of the labeling patterns as indicating a shut-down in RNA synthesis. Perhaps what was being changed was our ability to measure incorporation into RNA rather than the synthesis of RNA itself.

In order to try to get around this, or at least get some idea of its significance, we did an experiment where we added excess label to a differentiating culture just before 16 hr. The first points were taken right after 16 hr when we knew we could get good uracil labeling in the RNA (Fig. 13). We then followed the distribution of the label as the culture went through the period when we no longer could get at it by pulsing precursors from the outside; thus, we pre-loaded the pools and then followed what happened afterwards. As you can see, the pools were labeled very rapidly. The value for the pool represents the difference between the total label and that in RNA. The counts in the pool increase rapidly, but you will notice that they go up until 17 hr and then the pool size drops

and the total counts decrease. All the loss of label from the plant can be accounted for by the label that reappears in the medium. Our interpretation is that part of the problem with uracil incorporation in our previous experiments was that we are trying to go against the system. During the stage from 16-17 hr, not only is the plant degrading RNA, as shown in one of the earlier figures, but the pools themselves appear to be actually shrinking at this time. Both factors would work against uracil penetration.

Figure 14 represents an experiment where we tried to circumvent this difficulty. I'm not entirely sure whether we did or not, but I think we did in part. The experiment was based on an assumption with which you may not agree and which we have to prove: that the "heavy" RNA was predominantly a ribosomal precursor. My argument will have validity only as far as this is true. We grew the cells as usual, adding C^{14}-uracil for 1 hr during the exponential growth to randomly label the whole-cell RNA. Then, as a function of time, we pulsed the cells with tritium-labeled uracil to see how much could enter RNA from the outside. The pre-existing, randomly labeled RNA inside the plants should give us some idea of the activity within the cells, particularly at the later stages when we could not get at it by pulsing from outside. As you can see (Fig. 15), the pattern of the randomly labeled RNA remains essentially constant throughout. However, the pulse labeling with tritium is very similar to our previous results with C^{14}-uracil: rather disperse labeling and a high-specific activity, heavy peak which completely disappears by 17-17½hr. No carbon^{14} activity appears in this region, even though we know from all our previous evidence that a considerable amount of this RNA is degraded. If one accepts the idea that the heavy peak is a

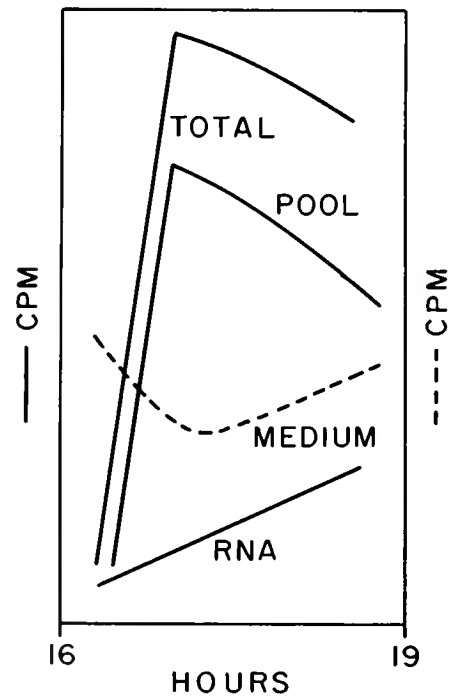

Fig. 14.

Incorporation and distribution of C^{14}-uracil label vs time.

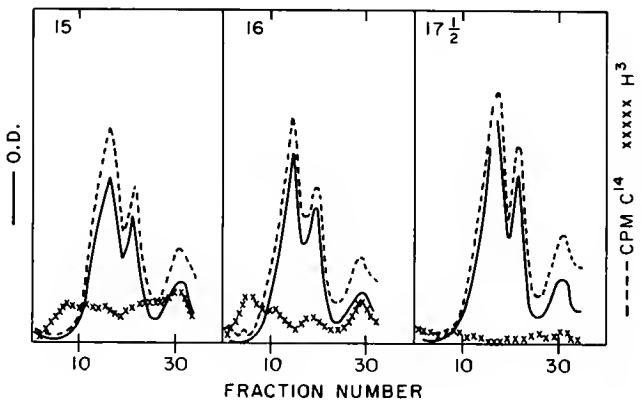

Fig. 15.

Sedimentation profiles of steady-state labeled (C^{14}) and pulse-labeled (H^3) RNA vs time.

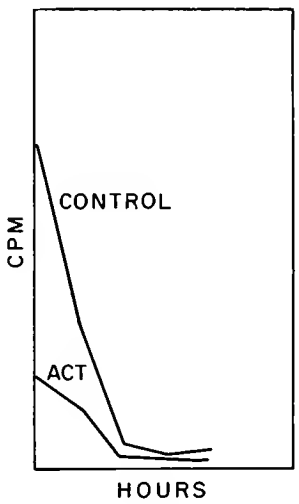

Fig. 16.

Actinomycin inhibition of uracil incorporation.

precursor for ribosomal RNA, this experiment can be interpreted to mean that there is very little, if any, turnover of RNA into new ribosomes. It is, however, all based on this particular argument which we have yet to prove.

I can summarize what I have said by pointing out that we did all this work, really, to prove that our original interpretation was correct. From what I have just said, as well as Turian's work with *Allomyces* (2, 7), it appears as though the ribosomes actually move and become aggregated in some way within the cytoplasm. I haven't any idea about the physical mechanism for accomplishing this and we are just starting to look at this stage in the development of the spores in the electron microscope.

Our results at this point suggest that new ribosomal RNA - and, apparently, transfer RNA - synthesis is not necessary for spore formation. Can we say anything about other kinds of RNA? Obviously the question of messenger RNA arises. Does the formation of the spores require production of messenger RNA and is there any way that we can show evidence for it? Our information on this is quite incomplete, but I would like to describe what we do know very briefly.

Actinomycin D is a very effective inhibitor of morphogenesis. At 25 μg/ml, the concentration we have normally used, actinomycin efficiently reduces the incorporation of uracil into RNA (Fig. 16). In this experiment the actinomycin was added at the same time as the uracil and even without pre-incubation it caused a 60-80% inhibition of incorporation. It is only effective on leucine incorporation if the plants are pre-incubated with the antibiotic. There is no figure for this, but at least 5 min of pre-incubation are required for significant inhibition of leucine incorporation.

Figure 17 shows the effect of two protein inhibitors on leucine incorporation, puromycin and p-fluorophenylalanine (PFP). Somewhat to our surprise, PFP, the lower curve, was much more effective than puromycin, although this could be, in part, a concentration effect. PFP, however, is also much more effective in causing morphological arrest (Fig. 18).

These graphs require some explanation. The experimental cultures are on the left while the control cultures on the right show the morphological progression of the parent culture. The top is for actinomycin treatment and the bottom is for PFP- and puromycin-treated plants. Samples were removed as a function of time from a synchronous culture, placed in inhibitor,

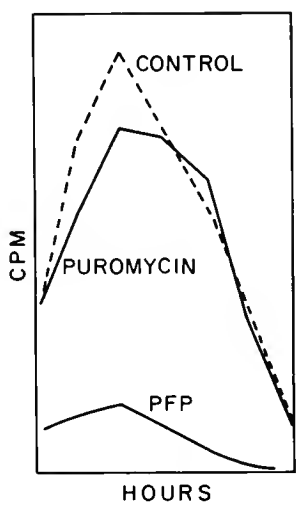

Fig. 17.

Inhibition of leucine incorporation by protein inhibitors.

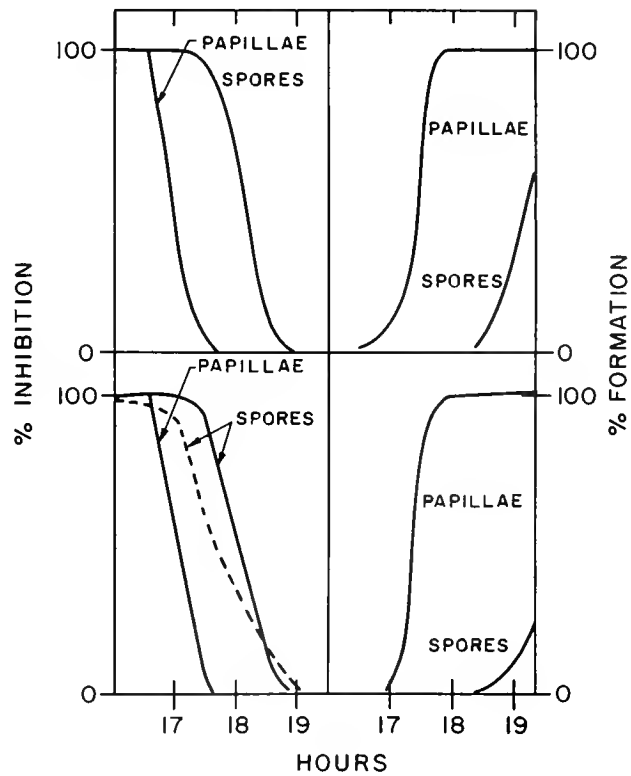

Fig. 18.

Inhibition of development vs time of treatment. Top left, actinomycin; top right, control. Bottom left, p-fluorophenylalanine (———) and puromycin (------); bottom right, control.

and allowed time to go through the entire differentiation process, in fact, well beyond when it had ended in the untreated controls. We then fixed all the samples and examined them to determine the percentage of cells reaching a recognizable stage. The curves on the right show the normal pattern of papilla formation in the control cultures and the release of the spores at the end of the experiments.

There was no inhibition of papilla formation by actinomycin at 17½ hr. That is to say, there is essentially no effect on the papillae if added just before the papillae actually appear, but if the actinomycin was added about a half-hour earlier, 16-16½ hr, then no papillae formed and it gave 100% inhibition. The same thing was true at a later point for spore formation, the obvious "cleavage" of the protoplast to form the individual spores. At 17½ hr it was 100% effective; by 19 hr it was completely ineffective. Essentially the same curve was obtained when we used PFP. If you accept the fact that these are well-synchronized cultures and notice that the control patterns are superimposable, then the experimental curves for actinomycin D and PFP are virtually superimposable also; puromycin is only indicated here for spore formation because of the 100 μg/ml concentration used it did not inhibit papilla formation. It did result in abnormal-looking papillae. They were long, multiple, and somewhat twisted in contrast to the short, single, and symmetrical papilla at the tip of the normal plant grown under our conditions. We do not know the reason for this effect. PFP, on the other hand, completely mimics the behavior of actinomycin.

GROSS: Do you know that the puromycin is doing what you want it to do?

LOVETT: No, we don't. This is really the only positive result that we have with puromycin, and I am not going to say any more about it.

CHALKLEY: In our work with tobacco cells, it shows a similar lack of protein inhibition. If the puromycin does get into the cells, then one may assume that there is some degradation of part of the molecule.

LOVETT: I'm glad to hear that, because we haven't yet tried other concentrations. We were not sure that we were using an adequate concentration and thought that it might not be getting in fast enough.

CHALKLEY: I think the problem is not unsolvable.

GROSS: I think the pea work is really much more dramatic.

LOVETT: It is interesting and also most unexpected.

B. WRIGHT: Is the time of effect of the actinomycin accompanied by permeability changes?

LOVETT: It is so effective in inhibiting uracil incorporation that I think this means that it is getting into the cells.

B. WRIGHT: Yes, but I mean the time at which it first starts to inhibit.

LOVETT: No, at least I choose to interpret it as meaning something about when it is acting. This is indirect evidence, to be sure. Before we see obvious morphological changes it stops everything. When it stops papilla formation, it has obviously stopped growth. However, the point is, if you add it later it no longer has any effect, even on papilla formation. It has no effect on papilla formation but will stop later development.

Returning to the fact that PFP acts like actinomycin on development, you will note that, as near as we can tell, it shuts down leucine incorporation almost immediately. It is an effective inhibitor. This, plus the fact that it mimics the actinomycin effects almost identically in the morphological progression, leads us to suggest that it may be acting in some way other than simple incorporation, as has been shown in *E. coli* (8), to form "nonsense" protein. I have heard rumors of a similar situation in another system. I think that inhibition by producing nonsense proteins would take some time unless there was *a* critical protein, and nothing could proceed unless it was functional. This could be true, but I am interpreting it somewhat differently.

GROSS: Well, does it shut down RNA?

LOVETT: We haven't done this yet, but I think it shuts down protein synthesis.

GROSS: It shuts down protein synthesis as measured by leucine incorporation?

LOVETT: We have only measured leucine incorporation so far, but on the basis of the fact that it is so effective on total development I think that it shuts down all protein synthesis.

The inhibition results suggest that the actinomycin effect is on RNA made half an hour before the papillae are formed, and that the necessary protein is probably also made at nearly the same time. This could explain why PFP mimics the effect of actinomycin. Though we have much to do before we can really prove it unequivocally, it is nicely consistent both in this case and in the case of the spore formation later on. To me, it suggests that we do not

have to invoke long-term messengers. We do not know what the papilla is composed of, but it looks as though it might be a polysaccharide of some kind. It may well be that some kind of enzyme or enzymes are made that begin synthesis long before one sees the morphological event itself. We have no reason to say that there couldn't be other kinds of RNA's having other functions being made at the same time; all we can point to is the observable event.

We consider this to be interesting preliminary evidence and it is the kind of problem we want to pursue with our system. We would particularly like to be able to characterize some of the RNA's we think might be produced. This, I'm sorry to say, just about summarizes all we know about spore diffferentiation.

Our information on spore germination is even less definitive. Because of some interesting parallels with the work discussed by Dr. Gross on the first day (9) I would like to mention this in an informal way. The next few plates will show several stages during the germination of the spore - a transformation fully as dramatic as the differentiation of the spores themselves. The next slide (refer back to Fig. 2) is just to refresh your memory with regard to the organization of the spore with its nuclear cap and the large mitochondrion, which, in certain sections, extends well up toward the anterior end of the cell. Germination occurs quite rapidly under our conditions and the next electron micrograph (Fig. 19) shows the first stage we have been able to catch. The spore is rounded up; it no longer has the very thin, delicate outer membrane and already looks as though it is beginning to form wall material. It has retracted the flagellum, and in this micrograph you can see a longitudinal section of the flagellum within the cell. The mitochondrium, instead of being localized as before, is in several parts of the cell and appears as more than one. All these events occur in a matter of a few minutes. Even at this early stage, there are points where the originally continuous nuclear cap membrane has become discontinuous. We do not know how it occurs, but the ribosomes are beginning to "leak out". Perhaps this is not the right term, but that is exactly how it appears.

Figure 20 is a section of a cell a few minutes later during germination. It is now a little round cyst with the ribosomes spread throughout the cell and no sign of a nuclear cap. There is the nucleus, a mitochondrion, which we think may actually have divided by this time, and a few of the granules from the zoospore. This cell

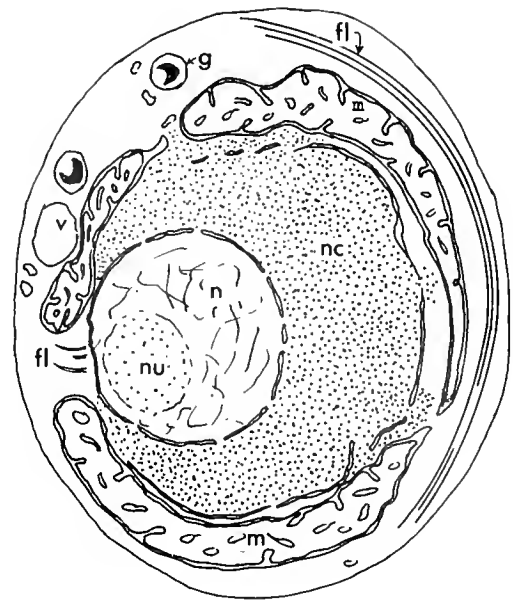

Fig. 19.

An electron micrograph of an early stage in germination to show the retracted flagellum and the beginning of nuclear cap disorganization.

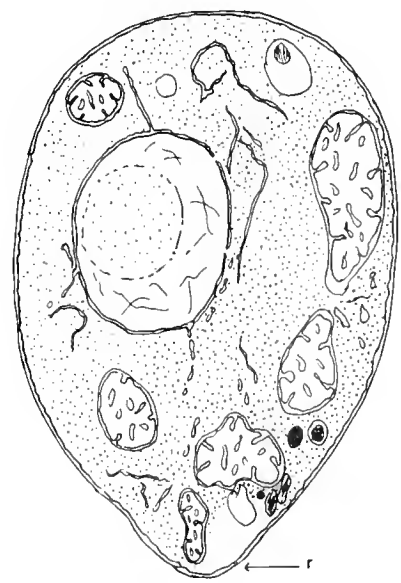

Fig. 20.

A very young stage to illustrate the complete dispersal of the cap ribosomes and initiation of the primary rhizoid (r).

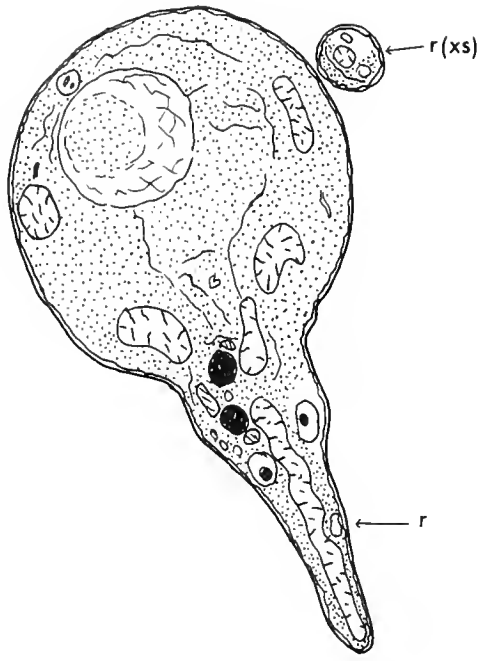

Fig. 21.

A young germling plant with a well-established rhizoid. xs, cross section.

has already started the process normally called germination; it has begun to grow out to form the primary rhizoid.

The next micrograph is a somewhat later stage (Fig. 21). It is a young germling after about 30-40 min in culture, which has the rhizoid well started. It is probably longer than it appears in the micrograph since a tangential section makes it taper down, but it could be the actual size. At this stage the germling looks like a perfectly normal cell and, in fact, development from this point involves mainly increase in size with branching and multiplicity of the rhizoids, in the absence of cell division.

DEERING: How big is this germling?

LOVETT: The spore body is about 7 x 9 microns, and this is roughly the same, about 8 microns in diameter before it starts to grow.

DEERING: What are the long, slender lines in the cytoplasm?

LOVETT: That is the endoplasmic reticulum. It is fairly prominent here but not at some other stages.

KAHN: Is that the nucleolus?

LOVETT: Yes, they have a large nucleolus that is always present. You remember seeing the spore? It was small and compact in the spore. I'll have more to say about it in a moment.

DEERING: In published results, Cantino mentioned that this plant seemed to separate into two cells.

LOVETT: That is not at this stage; it occurs later, during the time when spores are formed at the end of the growth phase.

DEERING: How much later is it? At what point can you really say that you've got division into two cells?

LOVETT: There is none at the stage I'm talking about. That occurs only at the end of the growth phase.

CANTINO: I didn't speak of cell division, only nuclear division.

DEERING: I'm talking about the two cells, the basal one and the one full of nuclei.

LOVETT: That is just before spore are released and is in the other experiments we were doing. I didn't discuss it because it is obscure and hard to see and we haven't really done anything with it.

We can easily get reasonably well-synchronized cultures of the germinating spores, at cell densities of about 10^9 cells per liter, all doing very nearly the same thing at the same time. The synchrony is not, however, quite as good as we have obtained from zoospore differentiation.

The next two figures illustrate the pattern of synthesis during early stages of germination. The cells appear to lose some dry weight during the first hour of germination (Fig. 22). They are so fragile, however, that we are sure some material was lost while trying to collect the cells and measure their dry weight. Thus, part of the drop may be artificial. After the first hour, the dry weight increases linearly for a matter of 6 or 7 hr at least. Figure 22 also shows DNA synthesis during the same stage, and you will notice that it increases nicely in a step function from one level to twice as much in a 2 hr period. Nuclear division, which still puzzles us, occurs during about the first hour of the 2 hr period of DNA synthesis. We are not sure what this means, but it may indicate that our culture is not as synchronized as it appears. This, however, remains to be seen. It does go through this first nuclear division in a reasonably synchronous fashion.

Figure 23 roughly illustrates the increase in total RNA and total protein during germination and early growth. Net RNA increase is not apparent for about 20 min, and new protein increase is not detectable until about 40 min.

Figure 24 is a summary diagram which we

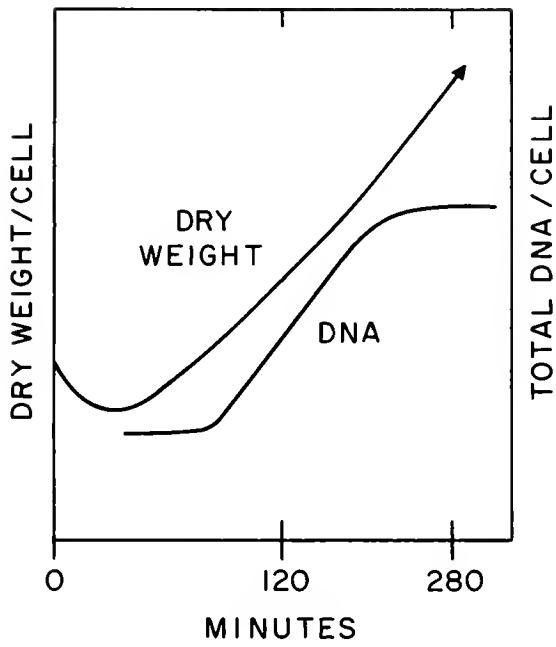

Fig. 22.

Dry weight increase and DNA synthesis in synchronous cultures of young plants.

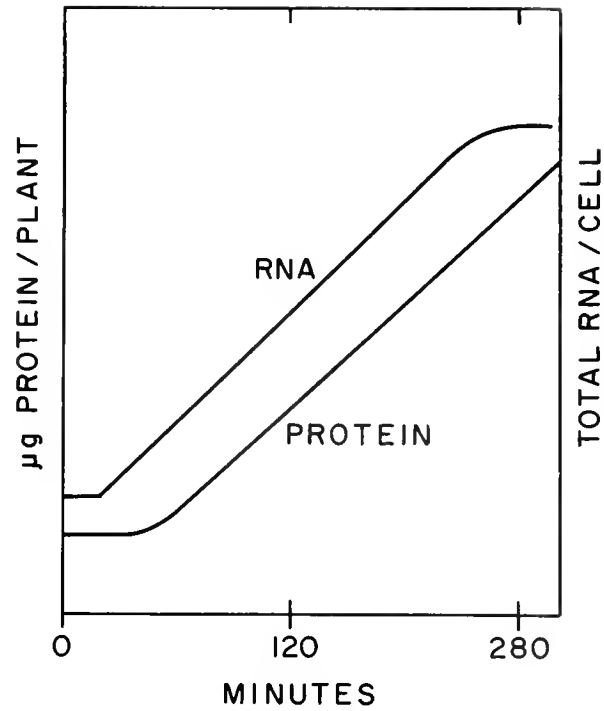

Fig. 23.

RNA and protein synthesis in synchronous cultures of young plants.

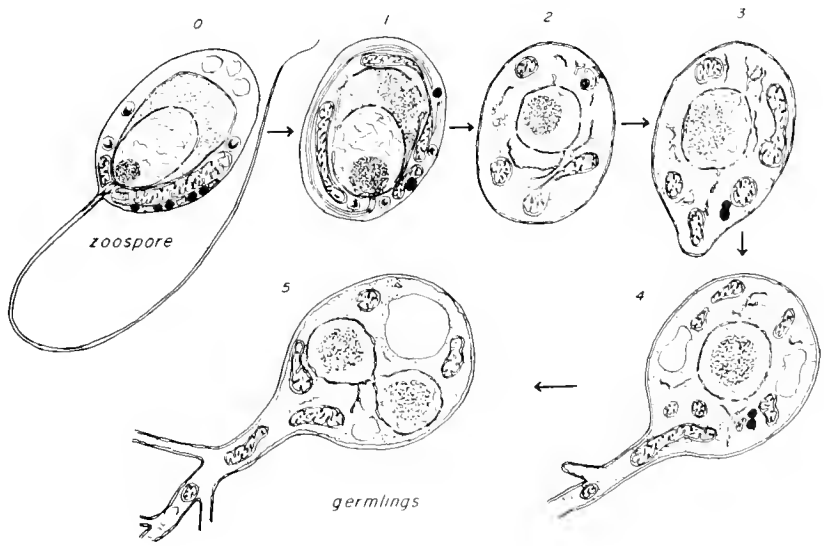

Fig. 24.

Stages in the germination and early growth of a zoospore. Stage 0, zoospore; stage 1, 12-15 min; stage 2, 20 min; stage 3, 25 min; stage 4, 120 min; stage 5, 240 min.

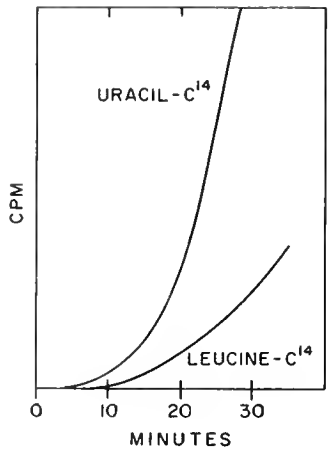

Fig. 25.

Precursor incorporation by zoospores germinating in an organic medium.

can use to try and interpret, or to fit into the morphological sequence, the points I have just discussed. Zoospore germination begins between 12 and 15 min after inoculating the spores into the culture medium (Stage 1). It doesn't occur sooner because the harvested spores are held on ice in very concentrated suspensions while being centrifuged and washed. Our germination experiments start when the zoospores are diluted up in the medium at 24°C. Twelve to 15 min later, they round up and begin to germinate. The next to last picture in the sequence (Stage 4) is a 2 hr plant with a well-developed rhizoid.

The previous slide showed that a measurable increase in RNA and protein cannot be observed until 20 and 40 min, respectively. The next figure demonstrates that incorporation of C^{14}-uracil or C^{14}-leucine can be detected much earlier (Fig. 25). Uracil incorporation occurs by 10 min, if not sooner, as does leucine incorporation, which here looks somewhat slower than it actually is because it has not been corrected for its differing specific activity. Such a correction would move it much nearer to the RNA curve.

While I have no further data to present, I would like to mention a few recent and interesting results. First, the pattern of synthesis and early differentiation of the spores - up to a stage where they form a tiny, uninucleate plant with a fairly long, branched rhizoid - will occur whether you put the spores in the growth medium or not. The spore appears able to accomplish this quite well at the expense of whatever it carries with it. The lipid and polysaccharide granules may serve as energy supplies for this. The same is true for precursor incorporation. Thus, the early events in germination appear to be nearly independent of the medium. The triggering of the spores to germinate may not be independent of the medium, but we do not know what sets the process in motion.

Now to go back to some of the problems I posed earlier concerning the function of the cap which we have obviously not really answered. As I said before, the reappearance of the ribosomes in the cytoplasm at germination could result from synthesis of new ribosomes at the expense of the cap ribonucleoprotein, or from migration of the pre-existing ribosomes. In the first case, ribosomal RNA synthesis would be required. The second might necessitate coding of the ribosomes for early protein synthesis, or it might require nothing. This is an area of great interest to us, and I would like to mention a few preliminary experiments which are not yet at the stage where I am ready to put them on a slide.

First of all, zoospore germination will proceed to between stage 3 and stage 4 (Fig. 24), when it has just produced a short rhizoidal outgrowth, whether it is in actinomycin D or not. In other words, if one adds 25-100 μg/ml of actinomycin the spore swims normally, settles down, rounds up, the cap breaks down, and the primary rhizoid is produced. At this point further development is completely inhibited. That is one observation. We know from other experiments that during the same period, 25 μg/ml of actinomycin is very effective in inhibiting uracil incorporation. We haven't looked at the RNA yet.

TS'O: At that stage is there any evidence that actinomycin is actually going in?

LOVETT: Well, I assume it goes in because it completely inhibits uracil incorporation which occurs at the same stage.

TS'O: However, can it still get in the later part?

LOVETT: It completely inhibits the later stage, so I assume it's getting in, though I don't have direct evidence. It is still inhibiting after that time, at least in control experiments, where we have had no actinomycin present earlier; by this I mean that it still inhibits uracil incorporation at the later stages. The third point is that actinomycin seems to have practically no effect on leucine incorporation during the first 30 min; so far, we haven't followed it much beyond that point. We have put the spores in 25 μg/ml of actinomycin, allowed them to germinate, and measured leucine incorporation, with C^{14}-

leucine present continuously. There was no difference between the incorporation in the control culture and the one with actinomycin.

Because of Paul Gross' results with embryos (9), I'd like to speculate even more: if, during these stages, we look at pulse-labeled RNA on sucrose gradients (we are just beginning to do this), the cells make RNA during the whole time, but it is not ribosomal RNA. The new RNA seems to be polydisperse, and in the spore it is very heterogeneous on gradients. It looks like a nice, classical bacterial messenger-RNA pattern. The new RNA does not match the ribosomal peaks. As soon as germination begins, we also get the same effect that Paul described for embryos - very "hot" labeling in the sRNA region. I was originally concerned that the RNA might be degraded and that something was wrong. I am only too happy to see that the same results were obtained with embryos. It isn't until actinomycin has its effect, at about 30-40 min, that we can show synthesis of ribosomal RNA. This suggests that during the first 30 min, the cell is operating with pre-existing RNA, that the spores, in fact, have been precoded for the earliest events. I say pre-coded because of the observation, not because we have direct evidence for it. It is just as mysterious to me as it is, perhaps, to those who work with sea urchins. We did look for polysomes and could not find them. After hearing Paul, I was struck by the fact that two cell types which, in part at least, have a similar function (to be non-synthetic and yet ready to begin synthesis at a certain time) seem to be so similar in their patterns of early RNA and protein synthesis.

KAHN: How long can a spore be maintained before it becomes inviable?

LOVETT: I've never really determined this, mostly because I've been interested in just the reverse. When we harvest our spores, we try to collect only those that have been discharged over a short interval. We then hold them on ice until the experiment begins, which means a matter of half an hour from the time we isolate them.

KAHN: Then zoospores can probably be kept a long time?

LOVETT: Well, actually they can swim almost for days.

CANTINO: I can answer that question fairly positively. Some spores can remain viable for at least 24 hr, probably 36 hr, as swimmers. They are getting their energy reserves from within, not without, because this can happen in water. We know they have a sizeable polysaccharide pool.

TS'O: Did I hear correctly? In the beginning you said that you think the ribosomes may be made in the cytoplasm?

LOVETT: No, what I was suggesting was that one of the ways the cap might have been formed was by synthesis of new ribosomes by turnover and retention of the newly produced ribosomes just outside the nucleus. Our evidence suggests that the ribosomes were made before the stage of cap formation. I didn't mean in the cytoplasm.

KAHN: Let me restate my question. Does the resistant sporangium, wherein I assume the nuclear caps (ribosomes) are being formed, remain viable for 24 or 36 hours?

LOVETT: I'm not sure of your question. These spores can't be held very long. They have a high metabolic rate and they eventually disintegrate if not allowed to germinate.

KAHN: I'm talking about prior to release. If the resistant sporangium is, indeed, "resistant".

LOVETT: The resistant sporangium is resistant, but this isn't the resistant sporangium we've been working with.

KAHN: I'm aware of that.

CANTINO: There are no spores in the resistant sporangium even when it is mature.

LOVETT: The resistant sporangium is going to produce spores. Ed Cantino knows a lot more about the form it is in than I do. I haven't looked at this.

GROSS: What is the function of this gamete? Is it just dispersion by swimming or is it storage for a long period of time?

CANTINO: The resistant sporangium is a thick-walled structure. It appears to tide the organism over unfavorably environmental conditions in nature. We've had R.S. sitting in the shelf for almost twenty years, now, practically dry, and some of them are still viable. As far as we know, there are no pre-formed spores in them. Spores are induced to form once the resistant sporangium is put in water. Then the protoplast cleaves up into spores, the spores swim out, and to all intents and purposes they are like the spores Jim Lovett uses.

LOVETT: That takes about 8 hr; the process I have described in zoosporangia takes 3 hr. Our cultures go a little slower than Ed's because of the way we treat them.

PAPACONSTANTINOU: I'm sorry I didn't get this. Did you mention where the new ribosomal synthesis starts?

LOVETT: New ribosomal-RNA synthesis

starts at about 35-40 min and this is the same time that actinomycin really inhibits development. The cells won't go beyond this stage. However, this doesn't necessarily mean that a lack of ribosomal-RNA synthesis is the problem. It could be that pre-coding was exhausted at that time and the cells would have to make new messenger. We don't have any idea yet.

PAPACONSTANTINOU: What is the status of the nucleolus at that stage?

LOVETT: I forgot to mention that. The spores have a very small, compact nucleolus always located at the base of the nucleus opposite the flagellum. In plants, the nucleolus may include almost 60% of the nuclear volume. From less than 10% of the volume of the nucleus in a motile spore, it goes to about 50-60% of the nuclear volume in an actively synthesizing germling. With actinomycin treatment, they stay small. However, we have not made any critical measurements and this is a very rough estimate.

PAPACONSTANTINOU: I was just talking about the experiment that came out about a year ago from the Massachusetts General Hospital where they isolated the nucleoli from *B. emersonii* I believe.

LOVETT: Well, they said they did. They didn't prove it by any manner of means.

PAPACONSTANTINOU: I was wondering about that. Also, they claimed that they isolated a specific DNA whose base ratio was complementary to the ribosomal-RNA.

LOVETT: We haven't done anything like this at all. I'm not able to interpret some of Dr. Comb's work because he doesn't describe his methods very completely, and I can't really evaluate it.

MAURER: Did you try cycloheximide as an inhibitor of protein synthesis?

LOVETT: No. We did try chloramphenicol and it had no effect, but it doesn't seem to be very effective with fungi in general.

McCARL: Was that graph you had on the board with the 4S peak the labeling?

LOVETT: Yes, this was the labeling from uracil during a short incubation of 5 min.

McCARL: This shows synthesis?

LOVETT: I don't know specifically. This is the same question that Paul has; we don't know whether it is addition of terminal groups, whether it represents new synthesis, or what. We have to get some of it out and look at it.

GROSS: Are you worried about end-labeling?

LOVETT: No, I can get cytosine very easily. That's no problem. In fact, I'm sure this is true.

EPEL: Concerning actinomycin effects, this could be added during the first 30 min of germination with no effect?

LOVETT: We can let them germinate in it.

EPEL: Have you pulsed actinomycin?

LOVETT: No, we haven't tried removing it. We've tried it where they germinate in it and we've tested it where we take them out of a normal culture as a function of time and put them in it. You get the same results either way; that is, actinomycin doesn't have any effect until a certain point and then they become sensitive.

EPEL: If you add actinomycin during spore formation they still form spores, but are these spores then capable of germinating?

LOVETT: Yes, they seem to be. We haven't really tested this by growing them, but they seem to be perfectly normal spores. I think that this means that all the important events have happended by that time. It is interesting that the "packaging" of the ribosomes is one of the very last events. It does make sense that they are used for protein synthesis and then packaged up when practically everything essential is done. This occurs at about 10 min before 19 hr, and it is very soon after 19 hr that the spores are actually released.

GRUN: There were some small particles in your electron micrographs that resembled amyloplasts in a vague sort of way. Do you know what I mean?

LOVETT: Are you referring to the little round ones?

GRUN: There were some little round things with a darker, fairly homogenous stain.

LOVETT: There are some little cup-shaped structures in vesicles that are present in approximately the right numbers to be the particles that stain with the Nadi reagent, as Ed reported some years ago. These apparently occur only in the spore, since we have seen them nowhere else. I have no idea as to their function.

CANTINO: Their number depends upon the kind of plant which formed the spores and it can be modified by environmental changes. (cf. F. C. Cantino and E. A. Horenstein, *Mycologia 48*, 443, 1956).

GRUN: They don't contain starch, do they?

LOVETT: I doubt it. I don't know, to be quite honest with you.

GRUN: They were near the membranes in the electron micrograph.

LOVETT: They're very characteristic look-

ing. They have a single membrane, and are a little diffuse, but, more often than not, a section through a cell looks like this.

GRUN: Yes, and there were others that seemed to have a small drop.

LOVETT: I'm not sure what you mean. There are other granules.

GRUN: They're similar to amyloplasts.

LOVETT: There are other granules that are polysaccharide, I'm sure. Ed's done the work on polysaccharides. We haven't. The other granules you see in between the lipid granules in the side body are, I am quite sure, polysaccharide. They stain with the PAS reagent.

DEERING: I'm still after the answer to a question that I asked earlier. There seems to be a conflict in what you said with regard to the spore development and the later picture that you drew on the OC sporangium.

LOVETT: Do you mean the basal cell?

CANTINO: Well, the basal part is just rhizoids at the germling stage.

DEERING: I'm trying to find out whether or not it is a separate cell that makes rhizoids. What about the basal cell and rhizoid formation?

LOVETT: The basal cell occurs about the same time the papillae form, I would say, Isn't that right, Ed?

DEERING: It's very late in development?

LOVETT: Yes, and it is so obscure that it is sometimes almost impossible to see without very careful microscopic examination.

DEERING: Sometimes if you look at these in earlier stages, you can see what looks like a small ridge.

LOVETT: However, you don't see that until they're really ready to go. Frequently, the basal cell is so small, if it is there at all, that you can't see it. You just see the rhizoids.

DEERING: Is this basal "cell" separated from the rest of it in electron micrographs?

LOVETT: Give me about six or eight months and I'll be able to tell you. We are going to look at these stages in the electron microscope, but we haven't done it yet.

GROSS: Are there any nuclei in that lower cell?

LOVETT: In the RS form, the lower cell forms at the time when everything is moving out. In the OC, I don't think we could say one way or the other, but probably not. These plants, after all, are converting all their protoplast into spores. When the spores are gone, there is no reason to leave anything behind, because the plant is dead. It's an empty hull.

CANTINO: That cross wall is laid down centripetally, from outward to inward, and as that happens, what little protoplasm is down there migrates upward and you're left with an empty cell; at least, we call it a cell *a la* Robert Hooke.

GROSS: That is a bit confusing because it doesn't sound like a cell at all.

CANTINO: It's a compartment, a separate compartment.

LOVETT: It is very striking in the resistant sporangium where it forms a big stalk. You can almost watch the material move out of the lower part.

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THE MOLECULAR ASPECT OF NUCLEIC ACID INTERACTIONS

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The underlying philosophy and the strategy of our research is quite different from that presented in this workshop so far. We are interested in solving the problem of biology from the standpoint of chemistry and the approach of chemistry, especially physical chemistry. The experimental system usually consists of simple models. The approach is analytical and quantitative. The conclusion is generally unambiguous and mechanistic in nature. This is the power or the characteristic of physical sciences. Our problems are, however, oversimplification and unrealism. The conclusion may not be relevant to the more complex situation in biology in which we are interested. The major challenge to our work, therefore, is the meaningfulness of our results to the central problems of biology. We have to walk on a tight rope. On one hand, the system has to be simple enough to be analyzed quantitatively from the standpoint of physical sciences. On the other hand, the system has to be complicated enough and sophisticated enough to contain the essence of the biological world. I hope to demonstrate to you how we try to meet both demands in our research.

From the standpoint of chemistry, study of the biological system can be viewed as the study of the structures, properties and interactions of biopolymers, with themselves and with small molecules. Of all biopolymers, proteins and nucleic acids appear to be the most important in terms of the specific interactions which lead to information transfer.

The significance and some of the general principles of the interactions of nucleic acids with one another are well known. These concepts and this knowledge have served as the foundation for the development of molecular genetics. We wish to reexamine quantitatively the basic principles and the nature of the forces which govern the interactions of nucleic acids, and to do so by physical chemical studies.

The problem is approached at three levels of complexity: (1) interaction in solution between the monomeric units of nucleic acid and their analogs and derivatives; (2) interaction between the monomeric units and the nucleic acid polymer; (3) interaction between nucleic acid polymers. The present review is confined to investigations at the first two levels.

We concentrate first on interactions of neutral compounds and thus avoid complications due to the strong electrostatic interactions of charged molecules. Because both the sugar and the phosphate moieties are common to all nucleotide units, specific interactions of nucleic acids must reside in the purine and pyrimidine bases. Therefore, the experimental approach may quite justifiably be focused on the interaction of uncharged bases and nucleosides and their interaction with nucleic acid polymers.

The first level of interaction includes the following problems: Does a solution of monomeric units, such as free nucleosides, interact within itself? To what extent? By what mechanism? To answer these questions, three types of physical-chemical measurements have been made using, because of solubility problems, pyrimidine nucleosides and purine.

Vapor pressures of solutions of purine, uridine, cytidine, 5-bromouridine, and 6-methylpurine, from 0.1 molal to approximately 0.8 molal have been measured thermoelectrically (1, 2). Osmotic coefficients, ϕ, were calculated from the data (Table I). Activity coefficients at 25° were calculated from the osmotic coefficients by the Gibbs-Duhem relationship using a computer which performed a

TABLE I

Molal Osmotic Coefficients[a] of Various Solutes Determined in Water at 25°[b]

Molal Conc.	Purine	6-Methyl-purine	Uridine	5-Bromo-uridine	Cytidine
0.05	0.917	0.786	0.969	0.948	0.967
0.10	0.849	0.682	0.943	0.894	0.935
0.15	0.794	0.624	0.921	0.844	0.905
0.20	0.749	0.582	0.901	0.801	0.876
0.28	0.714	0.544	0.883	0.766	0.850
0.30	0.685	0.510	0.866	0.738	0.826
0.35	0.662	0.484	0.849	0.715	0.804
0.40	0.643	0.469	0.833	0.693	0.785
0.45	0.627	0.461	0.817		0.763
0.50	0.614	0.456	0.801		0.752
0.55	0.601	0.446	0.786		0.738
0.60	0.590	0.427	0.773		0.724
0.65	0.578	0.407	0.762		0.710
0.70	0.567	0.410	0.755		0.695
0.75	0.555				
0.80	0.544				
0.85	0.532				
0.90	0.522				
0.95	0.521				
1.00	0.505				
1.05	0.501				
1.10	0.501				

[a] Data from Ts'o, Melvin and Olson, *J. Am. Chem. Soc. 85*, 1289 (1963) and Ts'o and Chan, *ibid. 86*, 4176 (1964); reproduced with permission of the American Chemical Society.
[b] These are fitted osmotic coefficients computed from the experimental values.

numerical integration on the fitted polynomials and related molal concentration to ϕ (Table II). The data clearly indicated that the properties of these bases and nucleosides in solution are far from ideal. Values of both osmotic coefficients and activity coefficients are much below unity. These results establish the concept that purine and pyrimidine nucleosides do interact extensively in aqueous solution. Recently we have extended this type of measurement to other less soluble purine nucleosides. The osmotic coefficients at 25° at a concentration of 0.1 molal in water of 2'-methyladenosine is 0.723, of 2'-deoxyadenosine, 0.668, and of N^6-methyladenosine, 0.548. Evidently, adenine nucleosides do associate in water even more extensively than purine and to about the same degree as the 6-methylpurine.

After further analysis for their congruence to different models for multiple equilibria, the thermodynamic data were found to be incompatible with the model which assumes that only dimers are formed. Thus, the degree of association of these compounds may go beyond the dimers stage to a higher degree of polymerization. Most of the results are consistent with the model which assumes that the association process continues through many successive steps (at least above five steps) with the same equilibrium constant. Comparison of the equilibrium constant and thus the standard free energy changes is given in Table III (1, 2). This table shows that the tendency of purine to associate is much greater than that of pyrimidine nucleosides, which in turn is greater than that of urea.

Now, what is the mode of association of these

TABLE II

Molal Activity Coefficients[a] at 25° Computed from the Fitted Osmotic Coefficients[b]

Molal Conc	Purine	6-Methylpurine	Uridine	5-Bromouridine	Cytidine
0.05	0.844	0.626	0.939	0.902	0.930
0.10	0.728	0.469	0.885	0.811	0.878
0.15	0.641	0.385	0.845	0.732	0.824
0.20	0.575	0.329	0.808	0.666	0.776
0.25	0.522	0.287	0.775	0.613	0.733
0.30	0.480	0.255	0.744	0.569	0.695
0.35	0.446	0.230	0.716	0.533	0.661
0.40	0.418	0.211	0.690	0.502	0.631
0.45	0.394	0.196	0.665		0.604
0.50	0.374	0.185	0.641		0.580
0.55	0.355	0.173	0.620		0.555
0.60	0.339	0.162	0.600		0.537
0.65	0.324	0.152	0.582		0.518
0.70	0.311	0.146	0.568		0.499
0.75	0.297				
0.80	0.286				
0.85	0.275				
0.90	0.264				
0.95	0.255				
1.00	0.247				
1.05	0.240				
1.10	0.235				

[a] Data from Ts'o, Melvin and Olson, *J. Am. Chem. Soc. 85*, 1289 (1963) and Ts'o and Chan, *ibid. 86*, 4176 (1964); reproduced with permission of the American Chemical Society.
[b] See Table I.

TABLE III

Summary of the Analyses of the Osmotic Data Based on Treatments of Multiple Equilibria[a]

	$\underline{K}$	$\Delta \underline{F}^{o}$	$\underline{n}$
	(Molal^{-1})	(-RT ln $\underline{K}$, cal.)	($\underline{K}_n = 0$)
Purine	2.1	-440	$5 > \underline{n} > \infty$
6-Methylpurine	6.7	-1120	$5 > \underline{n} > \infty$
Uridine	0.61	+290	...
Cytidine	0.87	+80	...
5-Bromouridine	$\underline{K}_1 = 1.0$	0	...
	$\bar{\underline{K}} = 2.9$	-630	$\underline{n} = 4$
Urea	0.041	+1190	...

[a] See Ts'o and Chan, *J. Am. Chem. Soc. 86*, 4176 (1964); reproduced with permission of the American Chemical Society.

molecules in aqueous solution? Do they associate with each other vertically through hydrophobic and stacking interactions, or do they associate horizontally through hydrogen bonding? These thermodynamic data do not support the hypothesis of horizontal association through hydrogen bonding for the following reasons:

1. Methylation and bromination enhance association.
2. All these bases and nucleosides associate much more extensively than urea which is one of the best hydrogen bonding agents in water.

More direct information about the mode of association of the bases and nucleosides in solution can be obtained by the study of nuclear magnetic resonance. It is well known that nuclear magnetic shielding is a very sensitive probe of inter- and intra-molecular interactions. In this case, vertical stacking interactions are easily distinguished from hydrogen bonding interactions and these interactions manifest themselves differently in the NMR. It is therefore hoped that the concentration dependence of the NMR spectra in aqueous solutions of purine and nucleosides will shed some light on the association mechanism. The NMR spectra of purine have been studied over the concentration range of .05 to 1 molar (3). Chemical shifts of the three protons in purine vs the concentration are shown in Fig. 1. A pronounced concentration effect has been observed. Proton resonances in purine are all shifted to higher fields as the solute concentration is increased. Shifts to high fields with concentration are well known for aromatic systems and are generally attributed to the magnetic anisotropy associated with the ring currents in neighboring molecules. Because of the mobile-electrons, a large diamagnetic current is induced in the plane of the ring by an external magnetic field when the field is perpendicular to the plane of the molecule. This ring current gives rise to a small secondary magnetic field which reinforces the primary field at the peripheral protons in the plane of the ring. In the region directly above and below the molecular plane, the two fields are opposed, however. As the concentration of a solution of aromatic molecules is increased, the average distance between molecules decreases and the protons of a given molecule will feel the secondary magnetic fields produced by the ring current of neighboring molecules. Since it is much more probable to find the molecules somewhere above or below the molecular plane of another aromatic molecule due to the dish-shaped nature of the aromatic molecules, this magnetic anisotropy of the ring current effect will lead to a high field shift with concentration or to a low field shift upon dilution. At higher temperature, or when the purine is dissolved in organic solvent such as dimethylsulfoxide and dimethylformamide, such concentration-dependent chemical shifts for the purine protons are greatly reduced. Furthermore, when the purines are protonated by hydrochloride so they cannot associate because of carrying a positive charge, such concentration-dependent chemical shifts are again practically eliminated. These data clearly suggest that the mode of association of purine is by the vertical stacking of rings in a partial overlapping fashion. As described above, the osmotic coefficients and activity coefficients of purine have been interpreted in terms of multiple equilibria and on this basis, populations of various associate species at varying concentrations were computed. Based on these population distributions of the associated species, we can calculate the concentration dependence of the chemical shifts which is also given in Fig. 1 (3). It can be seen that the calculated value and the experimental value are in satisfactory agreement. Therefore, a numerical correlation between the NMR data and osmotic data has been successful in

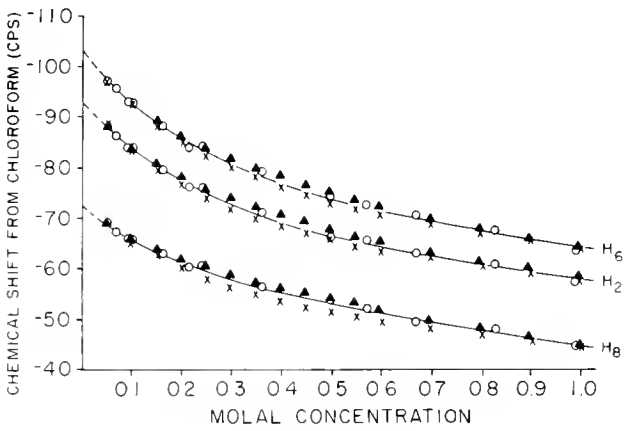


Fig. 1.

Concentration dependence of the proton chemical shifts for purine in aqueous solution at 25° (corrected for bulk susceptibility); shifts measured from external chloroform reference: —o—, experimental values; —X—, calculated values from overall average model; —▲—, calculated values from statistical partial-overlapping model. (From Chan, Schweizer, Ts'o and Helmkamp, *J. Am. Chem. Soc. 86*, 4182, 1964; reproduced with permission of the American Chemical Society.)

the sense that they reinforce and support the interpretations of each other.

Similar results have been obtained from the purine nucleosides, especially the adenine nucleosides series. In the case of 2'-0 methyl-adenosine, 2'-deoxyadenosine, and 6-methyl-adenosine, the concentration dependence of the chemical shifts is even larger than that of the purine. In all these cases, the H-2 proton of the 6 member ring of the adenine is shifted to the higher field than the H-8 proton in the 5 member ring. This indicates that the 6 member ring of the adenine does participate to a greater extent in the stacks than the 5 member ring of the adenine nucleosides. The pentose protons of H-1' are also shifted considerably to higher fields when concentration is increased while the pentose protons of the H-5' are hardly affected. As one proceeds around the pentose ring from the C-1' to the C-5', there is a progressive drop or decrease in the magnitude of these concentration-dependent chemical shifts. This indicates that adenine nucleoside interaction is preferentially localized at the purine base of the nucleoside so that the ring current magnetic aniosotropy is principally felt by the base protons. From this type of study, therefore, not only can we obtain the general picture about the mode of association, we can even get down to the detailed molecular structure of the stacks.

Currently, we are also working on the association of the nucleotides by vapor pressure osmometry as well as by nuclear magnetic resonance. In this case we have relaxed our restriction on the electrostatic effect of the phosphate group and have included this effect as a part of our model system with increasing complexity. Very interesting observations have been made. For instance, preferential interactions of the phosphate group with certain base protons of the nucleotides have been observed which have never been suspected before. Associations of base nucleosides and nucleotides have also been independently studied by Jardetzky (4).

The experiments detailed above concern solutions containing only one kind of solute. They concern, then, the interactions of the purine or nucleosides with themselves. What are the interactions between different compounds, for example, between a purine and a pyrimidine? The increase in solubility of the sparingly soluble adenine and thymine caused by the presence of highly soluble purine and nucleosides was adapted as the method for investigation of this type of interaction. As shown in Table IV the solubilities of adenine are much enhanced by the presence of purine. The enhancement is moderate in the presence of cytidine, uridine or pyrimidine and is practically nil in the presence of cyclohexanol, adonitol and urea. Similarly, the solubility of thymine (Table V) is enhanced by the purine and to a less extent by uridine and cytidine. These data were also analyzed by the treatment of multiple equilibria. The assumption in the treatment is that the bases interact to the same extent with the free and the associated forms of the interactants. Equilibrium constants

TABLE IV

Solubility of Adenine in the Presence of interacting Compounds [a]

Compounds Added	Concentration, molar	Solubility S molar x 10^3	
	(A) 25.5°		
None	....	8.25 ± 0.30[b]	
Purine	0.19	22.8	1.1[a]
	0.39	37.9	1.6
	0.58	52.6	2.9
Cytidine			
	0.18	15.6	1.6
	0.36	22.3	1.5
	0.54	28.7	1.6
Uridine	0.18	14.5	0.9
	0.36	22.3	1.5
	0.54	30.9	1.2
Pyrimidine			
	0.20	11.1	0.23
	0.40	15.8	0.58
	0.60	19.6	0.52
Phenol	0.20	12.0	0.50
	0.40	19.0	0.74
Cyclohexanol	0.20	9.47	0.15
Adonitol	0.60	9.84	0.39
Urea	0.60	8.88	0.36
	(B) 38°		
None	....	13.9	0.88[b]
Purine	0.193	29.1	1.33
	0.386	46.3	1.63
	0.58	60.9	1.70
Uridine	0.09	15.9	1.50
	0.18	20.1	1.26
	0.27	24.3	1.20
	0.36	30.5	1.33
	0.45	34.9	1.48
	0.54	42.7	1.11

[a] Data from Ts'o, Melvin and Olson, *J. Am. Chem. Soc. 85*, 1289 (1963); reproduced with permission of the American Chemical Society.
[b] Standard deviation.

TABLE V

Solubility of Thymine in the Presence of Interacting Compounds at 25.5° [a]

Compounds Added	Concentration, molar	Solubility S molar x 10^3	
None	....	27.4	± 0.70[b]
Purine	0.095	33.5	1.20[b]
	0.19	40.2	0.87
	0.39	49.8	1.10
	0.58	56.3	1.03
	0.77	64.0	1.50
	0.97	70.7	2.10
Uridine	0.18	33.4	0.70
	0.36	39.7	0.95
	0.54	43.7	1.27
Pyrimidine	0.10	29.8	0.40
	0.20	32.6	0.55
	0.40	37.4	0.80
	0.60	41.2	1.50
	0.80	44.6	1.67

[a] Data from Ts'o, Melvin and Olson, *J. Am. Chem. Soc. 85*, 1289 (1963); reproduced with permission of the American Chemical Society.
[b] Standard deviation.

for such interactions between different compounds have been calculated (Tables IV and V) and the general conclusions can be summarized as follows:

Interactions between purine and purine are stronger than the interactions of purine and pyrimidine, which are in turn stronger than the interactions of pyrimidine with pyrimidine.

The cross interaction of the pyrimidine nucleosides such as cytidine, thymidine and uridine with purine or other purine nucleosides can also be studied by nuclear magnetic resonance. In contrast to the large concentration-dependent chemical shifts previously reported for the proton resonance of the purine or purine nucleosides, the concentration dependent-chemical shifts of the pyrimidine nucleosides themselves are negligible (5). It is because the pyrimidine nucleosides are non-aromatic in nature and therefore do not support ring currents as do the aromatic purine bases. Therefore, the self-association of pyrimidine nucleosides cannot be monitored by proton magnetic resonance via the effect of the ring current magnetic anisotropy. However, the proton resonance of the pyrimidine nucleosides was found to be greatly affected by the purine due to cross interaction. Table VI summarizes the gross purine effect upon the protons of the pyrimidine nucleosides. A more detailed presentation of the data for the thymidine protons is given in Fig. 2 (5). Marked upfield shifts are noticed particularly for the base protons and anomeric protons H-1'. This effect falls off progressively as the proton distance from the ring increases. The direction of the purine-induced shifts plus their variation with distance for the respective protons from the apparent site of interaction suggests that the interaction is that of vertical ring stacking of the pyrimidine and purine bases.

The analysis of NMR data on solution properties of the monomers points the way for further studies of nucleic acid by this technique. For instance, an extensive NMR study of the

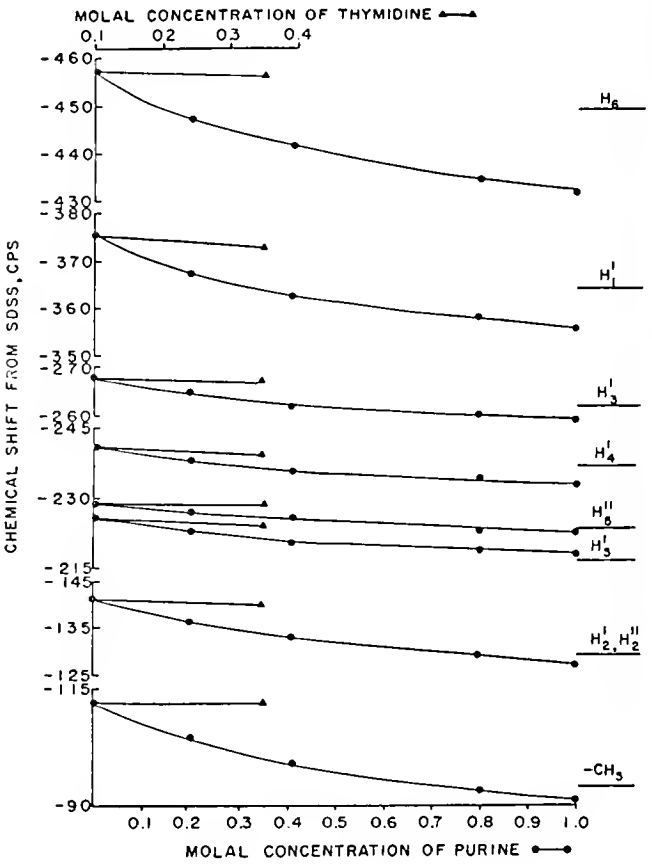


Fig. 2.

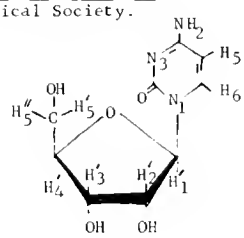
Chemical shift dependence of thymidine protons upon thymidine (— ▲ —) and upon purine concentration (— • —) at 35° in D_2O. Shifts measured from external SDSS. Magnetic field increases from top to bottom along ordinate. Spectra obtained at 60 Mc. (From Schweizer, Chan and Ts'o, *J. Am. Chem. Soc. 87*, 5241, 1965; reproduced with permission of the American Chemical Society.)

TABLE VI

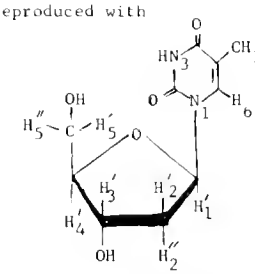
Concentration Dependence of Chemical Shifts for Cytidine, Thymidine, and Uridine Protons at 35° [a, c]

Concn., m		Chemical shifts from SDSS, c.p.s.							
		H-5	H-6	H-1'	(H-2',H-3',H-4')			H-5'	H-5''
A. Cytidine									
0		363.5	469.8	354.3	252.8			230.0	233.8
0.78 [b]		359.5	467.4	352.5	251.3			230.0	233.5
	Δδ	+ 4.0	+2.4	+1.8	+1.5			0.0	+0.3
		CH_3	H-6	H-1'	H-2',H-2''	H-3'	H-4'	H-5'	H-5''
B. Thymidine									
0		111.8	457.5	376.4	141.8	268.3	241.2	226.4	228.7
0.35 [b]		111.8	456.9	373.9	139.8	267.3	239.2	224.2	228.7
	Δδ	0.0	+0.6	+2.5	+2.0	+1.0	+2.0	+2.2	0.0
		H-5	H-6	H-1'	H-2'	H-3'	H-4'	H-5'	H-5''
C. Uridine									
0		353.0	469.7	353.8	259.7	253.2	246.9	228.0	233.4
0.77 [b]		350.1	468.2	350.9	257.6	251.9	245.1	226.7	230.9
	Δδ	+2.9	+1.5	+2.9	+2.1	+1.3	+1.8	+1.3	+2.5

[a] Solvent D_2O. Numbering of the nucleoside atoms shown, e.g., with cytidine and thymidine.
[b] Concentrations approaching limits of solubility.
[c] From Schweizer, Chan and Ts'o, J. Am. Chem. Soc. 87, 5241 (1965); reproduced with permission of the American Chemical Society.



cytidine



thymidine

dinucleotides has been made successfully in our laboratories. Hopefully, this approach can be applied to small nucleic acids such as transfer RNA (6). More complete description and discussion of these studies can be found in the original papers published from our laboratory. Nevertheless, sufficient data have been presented here to indicate the tendency for the bases and nucleosides in water to stack up vertically with the heterocyclic rings in a partial overlapping fashion. We are now ready to go to the next step of complexity.

The simple system of monomer-monomer interactions can be studied quantitatively by thermodynamic and spectroscopic methods. However, the system does not have the specificity exhibited at the level of polymer-polymer interaction. Consequently, we turn our attention to the nucleic acid interaction at the polymer-monomer level. A model system for this kind of study should have the following characteristics:

1. The polymer should have a minimal degree of self-interactions.
2. Solubility of both polymer and monomer should be sufficiently high.
3. The electrostatic forces should be minimal.
4. Its properties are relevant to those of a well-characterized polymer-polymer interaction system.

The above criteria are apparently met by the system: poly uridylic acid and adenosine (7). The binding of adenosine to poly U was first

studied by equilibrium dialysis at 5°. When the fraction of the occupied poly U binding sites is plotted as a function of free adenosines (Fig. 3), the resulting adsorption isotherm shows a very steep transition. No binding was detectable until a critical threshold concentration of adenosine was reached. This steep curve of adsorption isotherm is analyzed by the following equation derived from lattice statistics based on the nearest neighbor interaction (8).

$$\left(\frac{\partial \theta}{\partial \ln Y}\right)_{\theta = \frac{1}{2}} = \frac{\exp(-W/2kT)}{4}$$

where θ is the fraction of sites occupied, W is the interaction energy of the nearest neighbor and Y is the function of absolute activity of the adsorbate ($\lambda = e^{u/kT}$) and the partitian function for a molecule of bound adsorbate, q; λq at dilute solution is equivalent to $K_0 M$, where K_0 is intrinsic association constant for 1 molecule of adsorbate with a single site and M is the molar concentration of free adsorbate. Therefore:

$$\left(\frac{\partial \theta}{\partial \ln M}\right)_{\theta = \frac{1}{2}} = \frac{\exp(-W/2kT)}{4}.$$

Estimation from the slope of the curve (Fig. 3, open circle) yields a value of 30-60. In equation 2 the W is calculated to be -5 to 6 Kcal/mole which is the stacking energy of adenosine upon pairing with 2 U of poly U. This is comparable to the value of -4.8 Kcal/mole or -7.5 Kcal/mole calculated for the stacking energy of poly dAT and poly dI:dBC respectively by Crothers and Zimm (9).

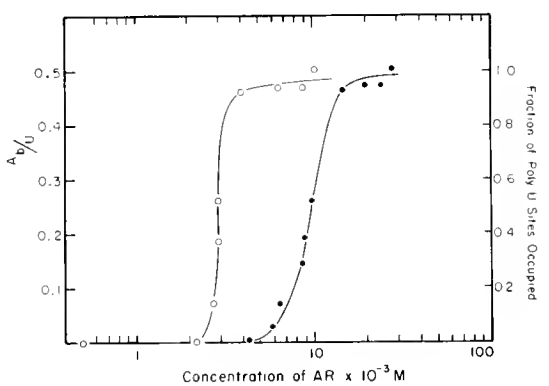


Fig. 3.

Adenosine bound per UMP of the poly U (1.5×10^{-2} *M*) versus adenosine input concentration at 5°C, 0.4 *M* NaCl, 0.01 *M* phosphate (HMP) (—•—). The fraction of poly U sites occupied versus free adenosine concentration under the same condition is given by —o—. (From Huang and Ts'o, *J. Mol. Biol. 16*, 523, 1966; reproduced with permission of Academic Press.)

Similar experiments were also performed using cytidine or inosine as the dialysable components. No detectable binding was found even at input nucleoside concentration as high as 2×10^{-2} *M*. Therefore, this interaction has the same specificity as the system of long chain polymers, i.e., the base pairing scheme of Watson-Crick. The stoichiometry of this binding reaction was studied by the solubility measurements, and it was found at low temperature that the stoichiometry is 2 U to 1 A, while at 20° the stoichiometry becomes 1 A to 1 U. The physical properties of this poly U-adenosine complex were further analyzed by sedimentation, viscosity, and by optical rotation measurement.

The formation of poly U-adenosine (AR) complex can be demonstrated by analytical ultracentrifugation. Sedimentation coefficients (S) of poly U in the absence (control) and presence of nucleosides are given in Table VII and the patterns are shown in Fig. 4. When N-6-methyladenosine, cytidine or inosine was mixed with poly U in equal amounts (1.5×10^{-2} *M* each) at 5° and 0.4 *M* NaCl, no change in either the pattern or the S value was found. As adenosine was mixed with poly U under identical conditions, a 33% increase in S value and a sharpening of the boundary was observed as compared with the control (Fig. 4). Similar results were obtained in 0.02 *M* $MgCl_2$ with the same mixture. In 0.4 *M* NaCl, as the temperature was raised, the percentage change in S value also increased to 43% at 10°C and 53% at 19°C, but it was accompanied by decrease in sharpness of the boundary (Fig. 4). The specific viscosities of poly U (1.5×10^{-2} *M*) and poly U-AR complex (1.5×10^{-2} *M* of each) in 0.4 *M* NaCl at 5° were respectively 0.602 and 1.05. As previously stated a parallel increase in S value (33%) has also been observed. The concurrent increase in both specific viscosity and the sedimentation coefficient of the poly U-AR complex as compared with those of poly U, unambiguously showed that there is a molecular weight increase in the polymer resulting from the complex formation.

Optical rotation measurement at 350 mμ was used to determine the conformation and

TABLE VII

Sedimentation of Poly U in Nucleoside Solutions [a,b]

Nucleosides	Buffer	Temp.	S_{20} control	S_{20} complex	% increase
Adenosine	0.4 M NaCl	5°C	4.68	6.21	33
Adenosine	0.4 M NaCl	10°C	4.03	5.78	43
Adenosine	0.4 M NaCl	19°C	4.00	6.11	53
Adenosine	0.02 M $MgCl_2$	5°C	4.70	6.45	37
L-adenosine	0.4 M NaCl	5°C	4.77	6.69	40
N-6-methyl-adenosine	0.4 M NaCl	5°C	4.77	4.77	0
Cytidine	0.4 M NaCl	5°C	4.06	4.06	0
Inosine	0.4 M NaCl	5°C	4.68	4.68	0

[a] Poly U conc. = 1.5×10^{-2} M
Nucleoside conc. = 1.5×10^{-2} M

[b] From Huang and Ts'o, *J. Mol. Biol. 16*, 523 (1966); reproduced with permission of the Academic Press.

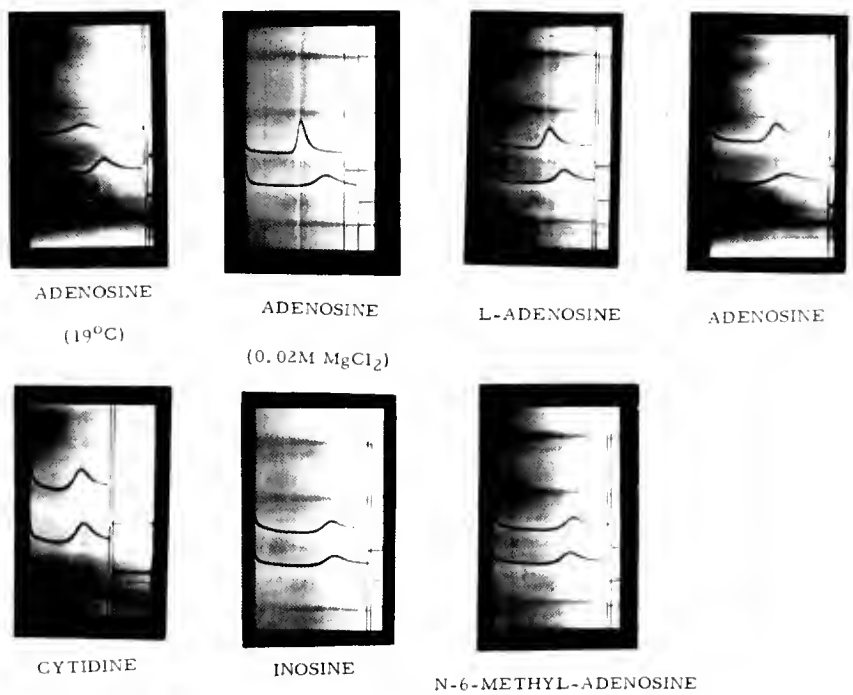


Fig. 4.

Ultracentrifuge patterns of poly U (upper pattern in each photograph) and poly U-nucleoside mixture (lower), each in concentration of 1.5×10^{-2} M, at 5°C and 0.4 M NaCl, HMP unless Indicated. (From Huang and Ts'o, *J. Mol. Biol. 16*, 523, 1966; reproduced with permission of Academic Press.)

stability of the poly U-AR complex. Poly U in 0.4 *M* NaCl gave a small positive rotation at low temperature (at 1.5 x 10^{-2} *M*, the observed rotation was about 0.2 degree at 5° C). The rotation decreased with increasing temperature, finally became temperature insensitive beyond 12° C as shown in the control curve in Fig. 5. On the other hand, 1.5 x 10^{-2} *M* adenosine alone gave an observed rotation of -0.09° calculated from the rotation at 1.2 x 10^{-2} *M* which was temperature independent. Nevertheless, where the two were mixed, a large increase in positive rotation was observed, +1.03° at 5° C. At the temperature insensitive region, the rotation of the mixture was the algebraic sum of its constituents. We took these to mean that the poly U-AR complex formed an ordered structure in 0.4 *M* NaCl and its stability was reflected by its melting behavior in response to the temperature variation. In 0.4 M salt, the optical rotation measurements remained essentially invarient with a temperature range from 0.5° C to 20° C. When poly U is mixed with cytidine, inosine or methylated adenosines no complex formation was observed (Fig. 5).

Formations of poly U-AR complex and its thermostability were highly dependent on adenosine concentration as illustrated in Fig. 6. When a constant amount of poly U (1.5 x 10^{-2} *M*) was allowed to interact with varying amounts of adenosine ranging from 3 x 10^{-3} *M* to 2 x 10^{-2} *M*, a saturation phenomenon similar to that observed in the equilibrium dialysis was also found, i.e., the magnitude of the maximum rotation and apparent stability remained unchanged after the ratio of input adenosine per UMP of poly U (denoted by A/U) reached unity.

Various analogs of adenosine were also tested for their binding capacity to poly U by the optical rotation and sedimentation methods with the expectation of obtaining information about an involvement of binding sites, and the role of the sugar moiety. The following compounds were tested: deoxyadenosine, L-adenosine (the pentose was L-ribose instead of D-ribose), (9 r-hydroxypropyl) adenine and (9-hydroxypentyl) adenine, (long-chain alcohols in replacing the sugar moiety). Complexing with poly U was found for all these four compounds. When the point of attachment of the purine ring was changed from the 9 position to the 3 position as in the case of 3-isoadenosine, complex formation could still take place. All these observations indicate that the sugar moiety of the adenosine does not play an important role for the binding. Optical rotation studies of the mixture of poly U with N-6-methyladenosine, with 1-methyladenosine and with tubercidin (A pyrrolo 2, 3-d pyrimidine riboside) revealed that no interaction took place. Therefore, the N-6-amino group of the adenineand with tubercidin (A pyrrolo 2, 3-d pyrimidine riboside) revealed that no interaction took place. Therefore, the N-6-amino group of the adenine appears to be definitely involved in binding with poly U. Other possible bonding sites are the N-1 and N-7 position of the adenine.

The two important aspects of the participation of adenosine in the interaction are its concentration dependence and specificity. The complex formation is undetectable in low nucleoside concentration. After a threshold concentration of adenosine is reached, the binding

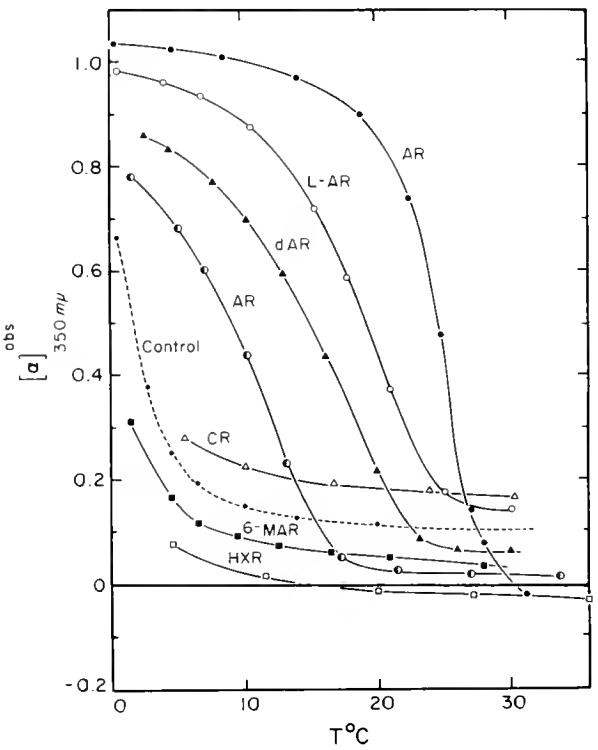


Fig. 5.

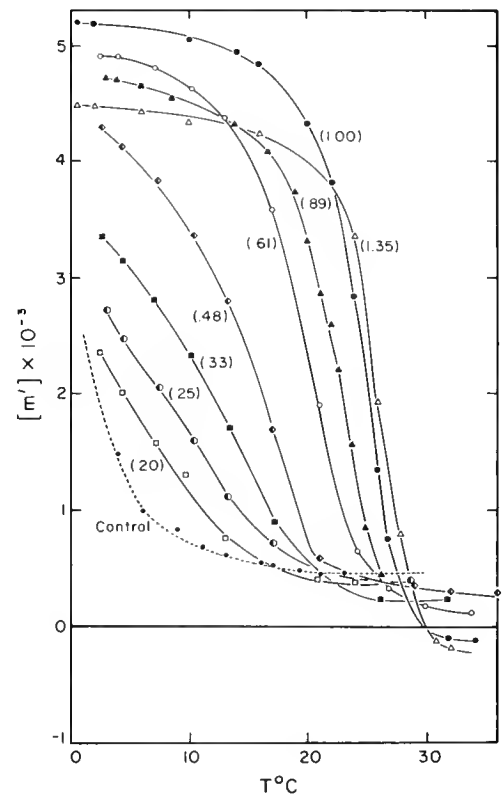
Observed rotation of poly U (1.5 x 10^{-2} *M*) - nucleoside mixtures versus temperature in 0.4 *M* NaCl, HMP. Concentrations of the nucleosides are: adenosine (AR), 1.5 x 10^{-2} *M* (—•—) and 7.3 x 10^{-3} *M* (– – • – –); L-adenosine (L-AR), 9.3 x 10^{-3} *M*; deoxyadenosine (dAR) 7.8 x 10^{-3} *M*; cytidine (CR), 1.1 x 10^{-2} *M*; N-6-methyladenosine (M^6-AR), 7 x 10^{-3} *M*; inosine (HxR), 1.5 x 10^{-2} *M*. (From Huang and Ts'o, *J. Mol. Biol. 16*, 523, 1966; reproduced with permission of Academic Press.)

increases rapidly in a cooperative manner until saturation. The key to the understanding of the interaction resides in the properties of nucleosides in solution of moderate concentration as detailed in the section of the monomer-monomer interaction. From these studies, we know that the stacking of adenosine occurs when the concentration increases. These stacks behave like the oligonucleotides, and therefore have much greater affinity to poly U than the free adenosine. At moderate concentrations, these associated stacks may serve as initiators for the subsequent binding of the adenosine molecule to poly U by a cooperative mechanism. In fact, the stability of most completely interacting complexes measured in our experiments is comparable to that obtainable for the poly U -trimer or tetramer (oligonucleotides) interaction. The forces responsible for stacking energy are short ranged. Calculation based on consideration of the nearest neighbor only gave an estimation of approximately 5 or 6 Kcal/mole as the free energy of stacking for this poly U-AR system. The results clearly indicated that hydrogen bonding cannot be the sole force responsible for the binding, since in dilute solution no binding is detected, even though hydrogen bonding capacity is still present. On the other hand, hydrophobic stacking forces alone do not allow the interaction to occur. Inosine, methylated adenosines and other adenine analogs probably all form stacks, yet they fail to bind to poly U. It appears, therefore, the hydrogen bonding and the hydrophobic stacking forces are both essential, with the former related to specificity and the latter related to stability.

Recently we have extended our investigations to the system of poly U-AMP interaction as well as to poly U-ATP and poly C-GTP interaction (10). In all these cases, the polymer and the monomer form an insoluble and stoichiometric complex in the presence of magnesium. We hope that the knowledge gained in this research will not only tell us about the physical chemical forces responsible for the structure of nucleic acids, but that it may also give us some idea about the mechanism of replication of nucleic acids in the polymerase system.

In conclusion, we have applied thermodynamic and spectroscopic methods to study the properties of monomers in solution. This research gives us knowledge about the extensive stacking interaction of the bases of nucleic acid in aqueous solution. Subsequently, we have applied this knowledge to the study of polymer-monomer interactions. Through this study, we have obtained certain important parameters and basic understanding about the forces responsible for the secondary structure of nucleic acids. Hopefully, the knowledge about these forces may also lead us to understand the mechanism by which nucleic acid replicates itself. Now, it appears that our chemical approach has reached the stage which is very close to being interesting to the biochemists, and perhaps even of interest to the developmental biologists.

So far, attention has been focused on the interactions of nucleic acids with themselves. Our laboratory is also starting to investigate the interactions between nucleic acids and proteins. Undoubtedly, research on this interaction will be of great importance in molecular biology and developmental biology. Interactions of purine with amino acids have already been



Flg. 6.

The melting of poly U-AR complex in 0.4 *M* NaCl, HMP measured by rotation at 350 mu. The poly U concentration is constant at 1.5 x 10^{-2} *M*. The parentheses indicate the input AR per UMP of poly U (A/U). (From Huang and Ts'o, *J. Mol. Biol. 16*, 523, 1966; reproduced with permission of Academic Press).

published from our laboratory (11). Results indicated that among all the amino acids, interaction of purine with tyrosine is the most important one. In the future, this approach may again guide us to the understanding of the interactions between two different types of biopolymers, namely, nucleic acids and proteins.

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THE PROBLEMS AND PROMISES OF RESEARCH ON THE MOLECULAR ASPECTS OF DEVELOPMENT
(Workshop Summary)

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We have spent three interesting and instructive days together. I have been much benefited not only by the talks presented in the formal sessions but also by the fruitful discussions with many of the participants in this workshop. We share a common feeling that at this moment we should review and reflect upon the problems and the progress in the field of developmental biology. Therefore, I shall present to you certain general consensuses and conclusions which we have reached as a result of our discussions. These conclusions are important to all of us for two reasons. The first is that experimental systems in the field of developmental biology are highly individualistic and specialized. Few investigators in the field can pick up helpful and specific experimental techniques for their own research by examining the work and the experience of others. In most cases each system has its own characteristics not shown by others. There are, to be sure, exceptions such as that demonstrated by the LDH-isozyme story lucidly presented by Dr. Edward Massaro. Experience and working knowledge learned from the isozymes' story can certainly be profitably extended to many other biological systems. The most important thing we can learn as a group is the underlying philosophy and strategy common to all of our research. The second reason is that we are continuously confronted by problems of communication, even as workers in the same field. There are biologists, chemists, physicists, biophysicists, etc., in this workshop. This is a very healthy sign reflecting the vigor and the promise of this scientific frontier even though it does bring its own problems. A review of the present status will help us with this aspect.

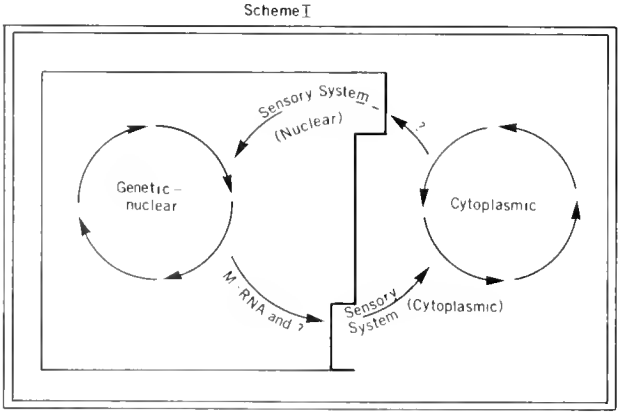
I will limit my comments mainly to the unicellular organisms and to a unicellular model. Dr. James Gregg and Dr. Arnold Kahn have presented to us some very interesting and relatively simple systems for the study of multicellular development. In these cases the importance of intercellular reactions must certainly be considered. In higher organisms, hormonal control plays a major role in development. Nevertheless, concepts developed from the unicellular model will provide the basis for further discussion of more complicated systems.

Differentiation of a cell clearly implies that the cell is suddenly doing something new, a diversion from what it was doing before. The degree of change, of course, depends upon the experimental system and the monitoring device. At present, however, it remains largely a question of semantics and will until we know more about the ground rules or the basic mechanisms common to all experimental systems. We will, in my opinion, soon reach some agreement that unless the change is sufficiently qualitative and distinctive we will not honor it with the name of "differentiation". Assuming agreement as to what is differentiation, now we are ready to pose the question, "What determines that the cell will change from its present course to a new one?". In this workshop we have loosely described this act as "the decision-making process or processes". I shall try to define and to clarify the concept of "the decision-making process" so that we can discuss it without undue confusion.

I would like first to define two decision-making bodies in the cell. Their existence is known from cell biology and biochemistry. The

first is the genetic-nuclear body shown in Scheme I. Within this decision-making body, namely the nucleus, there are many interlocking, interlinking, rate-dependent and rate limiting-processes. These processes are connected together in clockwise fashion as pictured in the scheme. The second "decision-making body" is the cytoplasm. In this "decision-making body", as well, there are many interlinking, rate-limiting processes which are shown connected in counter clockwise fashion in the scheme to indicate that they may be different from those in the nucleus. We now must set up a communication system between these two decision making bodies (Scheme I). Let us imagine a sensory system for the nuclear body which receives the cytoplasmic signals. We will similarly imagine a sensory system for the cytoplasmic body to receive the signals from the nucleus. These sensory systems can have varying degrees of sensitivities to various signals at a given time so as to allow certain information to be transmitted with great efficiency while other information is not transmitted at all. Thus, the communication system between these two decision-making bodies is controlled by the selectivity in transmission as well as by the regulation in generation of these signals. We do not have much information about the biochemical nature of these signals or about the sensory systems. This is certainly one of the most important problems of cell biology as related to developmental biology, i.e., "What is the biochemical nature of the communication network between the nucleus and the cytoplasm?". Recent research in molecular biology has indicated that one kind of signal which goes from nucleus to cytoplasm is the messenger RNA. The production or the transmission of this signal can be blocked by inhibitors such as actinomycin-D. The use of this inhibitor has provided us with much needed information about communication via m-RNA as evidenced in many of the talks given here. As to the biochemical nature of the cytoplasmic signals to the nucleus, the following experimental systems may be useful: hormonal control of protein synthesis in higher organisms; inductive enzymes formation in bacteria; and perhaps antibody formation in response to antigens. Research in these areas is of the utmost importance.

Scheme I



Decision – making Bodies Inside a Cell

Because of problems of presentation, Scheme I is drawn in an awkward manner as a reminder that the cell has spherical rather than bilateral symmetry. This has an important consequence. An external stimulus can not enter the nuclear region without passing through the cytoplasm. Nucleus and cytoplasm undoubtedly differ in sensitivity to external stimuli. Some stimuli may be more harmful to the nucleus than to the cytoplasm even though the stimulus has first passed through the cytoplasm. Nevertheless, we should remember that external effects on the cell always pass through the cytoplasm and therefore are subject to possible control from the cytoplasm.

The elegant experiments of Dr. James Gregg presented in the workshop and the discussion thereafter, enables us to assume for the moment that in order for this kind of decision making process (Scheme I) to take place no cell division is necessary. Thus, the barrier and the sensory systems between the nuclear and the cytoplasmic bodies need not be torn down in order for the decision to be made. This, of course, may not be true in all cases but the assumption will at least simplify discussion of our scheme. We have a cell which is going along in a dynamic state. Suddenly, it receives a new challenge or it reaches a certain state. It then makes a decision to embark on a course different from its original one. Where is the location of the decision making process and what is its pathway?

I shall try to describe three different pathways by which the decision making process might take place. The first I shall call the genetic-nuclear-determinant path. In this case, there is only one dominating influence in the cell. The decision is made in the nucleus in accordance with preinscribed genetic program and all the cytoplasm can do is to listen to the command of the nucleus. The best biological

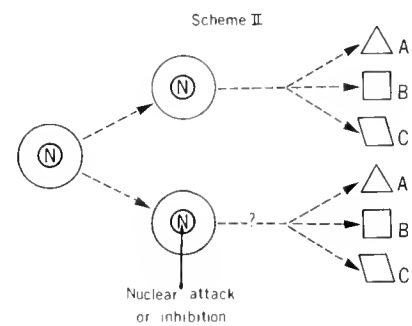
example of this case is the nuclear transplantation experiment done by Dr. Hämmerling's group between *Acetabularia mediterraneae* and *Acetabularia chronata*. These experiments demonstrate very clearly that the morphology of the algae is governed by the nucleus. Here we have a direct demonstration of the dominant role played by the nuclear body in differentiation.

The next pathway, another extreme case, will be called the cytoplasmic-determinant case. In this situation the cytoplasm is making all the decisions that are necessary and requires no help from the nucleus. Experimental demonstration of this situation is to destroy the nucleus or to block its pathways with an inhibitor such as actinomycin-D. Of this situation we have two subclasses. The first we shall call the latent message case. Certain messages are stored in the cytoplasm which are nuclear in origin. These messages are latent in the cytoplasm and will be called upon later when needed. It appears that this is the case in the development of sea urchin eggs as described by Dr. Paul Gross. Perhaps to some extent a similar mechanism is operating in the system described by Dr. James Lovett. A detailed discussion with Dr. Gross revealed that the result really depends on the monitoring system. In a superficial examination of the morphological appearances or general biochemical data, the influence of the nucleus may not be detectable, but by detailed biochemical analysis, as Dr. Gross has explained to me, in the case of protein synthesis the nucleus can be shown to exert a considerable control over the cytoplasm. The information originates in the nucleus, but the cytoplasm does have the power of control of the expression of this information until the right time.

The next subclass is the absolute cytoplasmic determinant case. The experimental demonstration of this type is hard to describe because it is foreign to our thinking on cellular biology. I cannot present any biological example of it but I can describe what the experimental requirement is in order to demonstrate its existence. We have a cell which can divide into two cells or more. Each of these new cells can make the decision to differentiate into various cell types as A, B or C shown in Scheme II. After blocking of the influence of these cells by direct destruction or by inhibitor, we see whether the cells can still differentiate into cell type A, B or C or not. The exciting results concerned with the stem cells described by Dr. James Till may provide an experimental system for testing of this case. It should be noted that this situation (Scheme II) is very different from that of the reticulocyte system. When cells reach the reticulocyte stage, their fate has been predetermined. Demonstration of Scheme II has to be done with cells which still maintain their capacity to choose among various paths.

It is not easy to separate these two subclasses, i.e., the latent message case from the absolute cytoplasmic-determinant case. Suicidal experiments with radioactivity decay of P^{32} or H^3 may be helpful. With proper experimental design, the latent message (if it is RNA) can be destroyed specifically while the rest of the cellular machinery is kept intact.

The last type of pathway to be described is likely to be the most common one, the perturbation-response system. In this case a perturbation, a challenge, arises (most probably) in the cytoplasm. It receives, for instance, a hormonal stimulus, it runs out substrates, or it is activated by an overdose of CO_2, or light, etc. In response, the cytoplasm transmits a signal to the nucleus as another perturbation. The nucleus, in response to this signal, picks up the preinscribed genetic program, and issues a new command for the cell to follow. Under these circumstances, the answer to the question about the exact location of the decision making body is debatable. Research workers who are primarily interested in the function of the genetic-nuclear apparatus, would say that the decision making body is in the nucleus, since it is the nucleus that issues the new command for the cell to change its course. Workers who are mainly interested in cytoplasmic events may claim that the cytoplasm should be called the



Experimental requirement for the demonstration of cytoplasmic determinant case

decision-making body since it is the first to receive the challenge and is also the first one to send out the request for change. We shall avoid these opinionated arguments by simply calling this category of decision-making processes the nuclear-cytoplasmic interdependent process.

This analysis brings out certain serious complications. These workers who have mainly been interested in the interlocking cycle located in the cytoplasm have no easy way of studying the biochemical nature of the cytoplasmic signals and the mechanism by which they are sent to the nucleus. This is simply because it requires a genetic-nuclear apparatus in order to detect these signals. If such investigators are not sufficiently careful they also may not be able to pick up the nuclear signals. In setting up the experimental condition to study the cytoplasmic cycle, the machinery of the cytoplasmic response to the nuclear signals may not be kept functional. Therefore, these workers may have unknowingly narrowed their point of view to only the cytoplasmic processes and completely neglected the important relationships and interdependency between the cytoplasm and the nucleus. Those workers who, on the contrary, have been mainly interested in the operation of the genetic-nuclear apparatus, may not be aware of the nature of the cytoplasmic signals, the origin of the cytoplasmic perturbation or the response of the cytoplasm to the nuclear command. Furthermore, our biochemical understanding of the nuclear events (the clockwise circle in Scheme I) is comparatively rudimentary. Reliable facts and concepts are few in this area and they are hard to get. For instance, we need to have a biochemical preparation of nuclear apparatus which can respond to cytoplasmic signals. Perhaps, such a nuclear preparation should synthesize new types of RNA when given a dose of hormone. Until we are sure about the nature of the cytoplasmic signals, it will, however, be very difficult to prepare such biochemical machinery responsive to these signals in an *in vitro* experiment. When the experimental result is negative, we don't know whether the machinery is nonfunctional or whether we have given the wrong signals. We are, however, encouraged by the effort and the results of Professor James Bonner's group in this direction as presented by Dr. Roger Chalkley.

In summary, the main theme we have discussed so far is not much different from the old idea in biology about a nuclear-cytoplasm relationship. However, we have redefined it in a context more adaptable to our time. In doing so, we have focused our attention on this relationship as the most important cellular factor to be considered in developmental biology. We hope this clarification will reduce the problem of communication and will provide a proper perspective about our own research as related to biology as a whole. Hopefully, this may lead to successful cooperation and fruitful exchange of ideas. We have an appropriate example in the workshop. The work of Dr. Edward Cantino is more related to the cytoplasmic events of the interesting water mold, *Blastocladiella emersonii*. On the other hand, the work of Dr. Lovett on this same organism is more concentrated on the function of the genetic nuclear apparatus. In putting the story together from their work, which undoubtedly they will do, we may be able to get a more complete picture about the intriguing mechanism of this creature in making its decision for differentiation and development.

Now let us look into the future for the next five years, say up to 1970. I think that the many basic problems of developmental biology in terms of biochemical hardware and mechanism are solvable to us in the next five years with sufficient manpower and financial resources. There is no sign of a shortage in either category. What I mean is that we do not need a technological break-through before we can solve these problems. For instance, we do not need to wait for the development of an electron microscopic movie camera. I think, rather, that the biggest barrier in fact is educational or communicational in nature. That is to say, the chemists working with a system will not know enough about the biology of the system and the biologists working on other systems will not know enough about their chemistry. Even in the field of biochemistry, those who work on nuclear events may overlook the crucial points related to cytoplasm and those who work at the cytoplasmic enzyme level may neglect the pertinent facts derived from the nucleus. We do need new ideas and ingenious approaches in these fields. These inspirations usually come from an organic synthesis of various disciplines not brought together before. This is why workshops of this type are so valuable.

Let's look ahead further into the coming ten years, say up to 1975. What will be thinking about? We saw a glimpse of that in this workshop. At that time, hopefully, we will know a

lot more about the various pathways. We probably will start studying the universality of these pathways as utilized by various organisms. For instance, we will like to know how generally the latent message mechanism is being used. It appears now that it is operative both in the sea urchin and in the water mold. We will start asking questions not only about how the genetic program is to be read (by that time we should really know what the genetic program is), but we may also ask, "How did the biological world derive this kind of program?" In other words, in the next five years we would like to link differentiation to molecular biology, and in the following five years we may wish to link differentiation to taxonomy and to the study of evolution. The presentation of Dr. Massaro about LDH systems in all kinds of fish and organisms is an early start of this type. By 1975 we may even have some answers from space study about Martian biology. By comparing Earthly biology to Martian biology, we may start to study developmental biology on different planets.

POLLARD: I'd like to comment in quite general terms on the message that may go from the cytoplasm to the nucleus. You can't have a message go from the cytoplasm to the nucleus that is physically too big. Almost certainly you're going to have to have something reasonably small which will get into the nucleus. The cytoplasm is unlike the nucleus, which has very large molecules moving out of it. However, to get something out of the cytoplasm into the nucleus you've got to actually drive it. It's like the old question of parking and unparking a car. If you park a car, you've got to put it in a fairly small place and it's fairly hard to do. If you unpark a car, you've got the whole world to go into. You can get big things out, but, I would suspect, you can only put small things in. So you've got to move from the cytoplasm to the nucleus such things as large proteins or ribosomes if a message is to be passed into the nucleus from the cytoplasm.

TS'O: Yes, in chemical biology we reach the same conclusion that you geneticists do: namely, that this would have a given direction of movement.

GROSS: I think that it's premature to decide now what informational macromolecules pass only in one direction.

POLLARD: Oh no, that's not what I meant.

GROSS: There is accumulating evidence that proteins as proteins can pass through the membrane into the nucleus.

POLLARD: Yes, they can pass in both directions. It is mainly the question as to what concentration might be necessary and what probability of passage would be. I would say that if you want something to diffuse rapidly and have a high probability of getting inside the nucleus with a message from the cytoplasm, while most molecules are moving out, there must be a high concentration gradient in that direction.

GROSS: It's feasible, however, that proteins that are present in low concentration in the cytoplasm as the result of some previous synthesis, under certain environmental stimuli might, for one reason or another, be carried into the nucleus independently of the concentration gradient.

POLLARD: Well, I would call that a "miracle".

GROSS: Well, it's possible that there are things that move only by diffusion, to be sure. However, there may also be carriers which themselves diffuse down a gradient.

TS'O: I would like to know some of Dr. Wright's opinions, especially after hearing her elegant biochemical experiments. Do you think such a general scheme is suitable for discussion?

B. WRIGHT: Well, I don't like the term "decision-making". I think it's so complicated that there are many "decision-making" processes interacting and interlocking. One simplified way you could look at it is in terms of the things that I've been concerned with which are going to be essential in all systems. Then you can have degrees of less and less criticalness. I mean, in order to get differentiation, as I said in my talk, you have to have immediate control at the level of substrates and enzymes. The whole small molecule milieu of the cell has to be keyed just right in order to accomplish this. Then, with respect to the time of the differentiation process, you have the tendency for less and less dependency in the criticalness of the control as you filter back toward the ultimate message.

TS'O: You would, nevertheless, say there are cases where the genetic control is a very predominant matter?

B. WRIGHT: At one time or another it is always of predominant importance. However, even when you can show, as with the nuclear experiment you mentioned, a very striking effect, all of these other levels of control have to be perfectly in shape in order to see

the effect of the genetic control on differentiation.

TS'O: Of course.

B. WRIGHT: I don't see any way to simplify this picture.

LOVETT: Isn't the important thing the relationship between how much of it is simple interaction between pre-existing systems and how much is new? A cell turns out a certain amount of enzyme as it grows at a certain rate for a certain amount of time. How much is just interaction between products in that kind of pathway and another pathway to which it connects? For instance, perhaps some of these products are going somewhere else and affecting enzymes because of their actual concentration level and thus preventing a pathway from functioning. Even though you might measure it *in vitro* and get a certain level, in fact, the enzyme isn't doing it nearly that fast because it's been shut off by some kind of a feedback mechanism. This could occur in the cytoplasm or by a circuitous path.

B. WRIGHT: This is pure speculation because there are practically no systems in which we have knowledge enough to do anything except point to *one* little effect somewhere.

LOVETT: Well, specifically, yes. However, there's certainly good enough precedent that small molecules having nothing to do with the function of a specific enzyme can cause things like inhibition. Why does one have to restrict it to a previous enzyme in the same pathway? Why couldn't it be used in a coordinating sense to regulate more than one pathway?

TS'O: Well, actinomycin is actually a good demonstration of this. Presumably, actinomysin-D doesn't affect enzyme activity.

LOVETT: Yes, but I think the best answer for that is some other experiments I have done with another organism where I am really worried about actinomycin effects: that is, it causes a lot of turnover of RNA when the cells are not making any protein.

STROTHER: You're talking about a single cell situation here, but even so it seems to me that the geometry must be taken into account. For instance, the nucleus is surrounded by a cytoplasm, in general, and the cytoplasm is surrounded by the external environment. Now, it would seem to me that what you're really missing in your overall schematic here is the interaction that you observe in a multicellular organism in the very close interaction, even in the single cell, with the environment surrounding it. I think you're missing a very important part with regard to your signals. Also, I don't know of any part of a consistent theory that doesn't involve statistical analysis of some sort, and I don't see where that appears here. Are they buried in the noise?

TS'O: Well, I'd like to take that in two parts. First, the membrane is of the greatest importance. Dr. Kahn, Dr. Gregg and myself have certainly talked a lot on that. We're very conscious of the membrane, but at the present I didn't have enough time to bring in all the elaborations.

The second point is that, as far as the noise level is concerned, you really couldn't argue this question unless you knew more about the details of this system. You'd have to know more about the hardware and the mechanics of doing it, before you could start posing questions of this kind. You need to know, for instance, when you make a protein, how much error you made in the process. Questions like that are beginning to be approached experimentally by Bob Loftfield and others, questions as to how often you make a wrong transcription. However, I don't think it's germane at this moment to put that into it. The point is not to make the system as complicated as possible but to keep it simple with enough essential parts to help our own decision-making in conducting our research.

GROSS: I'd like to add to both your responses. First of all, it's clear what we're really trying to do is decide what we mean when we say something is differentiated. Embryologists have really not been able to agree on that. Eventually this might lead to agreement; it might not. Now there are, in fact, systems such as this one cell which, in the absence of other cells, will do what we agree is differentiation. A single sea urchin egg, isolated from all other eggs, can be fertilized and, presumably, it will develop. I suspect that a single spore of *Blastocladiella* will germinate. So, to this extent, it's reasonable as a first approximation to talk about this rather simply.

The statistical point is a very good one and it's precisely there that I would locate all the considerations that motivate Barbara Wright's remarks because the noise level in this system represents the degree of deviation of the overall result of metabolism from the norm of the population as a whole. There's no doubt that in the cells there are momentary but significant fluctuations. It's the delicate interplay of all these separate steps in the pathway that prevents those fluctuations from becoming large

scale, unless a signal arrives from some place that says now the pattern must change. What we really want to know is, what are those signals? How do they work?

TS'O: Very well said.

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